We do not receive wisdom; we must
discover it for ourselves after a journey
that no one can take for us or spare us.

- Marcel Proust

University of Alberta

ADULT STEM CELLS AND PANCREATIC BETA CELL DIFFERENTIATION

by

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ABSTRACT

Type 1 diabetes, characterized by abnormal glycemic control, results from the destruction of beta cells within the pancreas of diabetic patients. Widespread islet transplantation, a clinical therapeutic option for type 1 diabetic patients, is limited by the shortage of donor islet tissue and a need for lifelong immunosuppression. For these reasons, alternate sources of islets are being investigated, as well as the impact of immunosuppressive drugs on these sources.

Mesenchymal stem cells (MSCs) derived from the bone marrow may potentially differentiate into pancreatic beta cells. Several differentiation strategies were developed, and their efficacies determined with respect to stimulating differentiation towards the beta cell fate. MSCs, induced to express the transcription factors ngn3 and Pdx1, and cultured with selective stimulatory growth factors, showed islet-like morphological and genetic changes. Cells formed spherical clusters and expressed the genes for Pax6, Nkx6.1, and NeuroD. These methods, however, did not result in cellular insulin.

Using a novel differentiation strategy to mimic the sequence of genetic events which occurs during normal pancreatic embryogenesis, we sought to enhance the differentiation of the MSCs. To achieve this, cells were transduced with multiple adenoviruses containing vectors for Pdx1, ngn3, NeuroD, or Pax6. *In vitro*, cells formed islet-like clusters and initiated expression of ngn3, NeuroD, glucagon, synaptophysin, Pax6, and Nkx6.1, however differentiation to the insulin-producing phenotype did not occur. Transplanting cell *in vivo* resulted in enhanced differentiation, with the detection of human c-peptide – a surrogate marker for insulin production.

Umbilical and mobilized peripheral blood may be MSC sources, and we investigated the efficacy of isolating MSCs from these two populations. Each population consisted of adherent cells with typical MSC phenotype, however these cells did not express classic MSC surface markers. Furthermore, cells transduced with ngn3 did not express ngn3 or other pancreatic transcription factors.

Finally, we investigated the impact of the immunosuppressive drug Sirolimus on the proliferation of pancreatic islets and ductal cells. We found that in human ductal cells, hypothesized to be adult pancreatic stem cells, and in neonatal porcine islets, which contain 57% ductal cells, that cell expansion was inhibited by 50% and 28% respectively.

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LIST OF ABBREVIATIONS

ABC	Avidin-Biotin Complex
AIRg	Acute Insulin Response to Glucose
ANOVA	Analysis of Variance
bFGF	Basic Fibroblast Growth Factor
BM	Bone Marrow
BMP	Bone Morphogenic Protein
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Mono Phosphate
cDNA	Complementary Deoxyribonucleic Acid
CK	Cytokeratin
CMV	Cytomegalovirus
CNS	Central Nervous System
c-peptide	Connection Peptide
DAB	3,3 diaminobenzidinetetrahydrochloride
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTPs	Deoxy-Nucleotide Triphosphate
DTT	Dithiothreitol
dUTP	Deoxy-Uridine Triphosphate
EDTA	Ethylenediamene Tetraacetic Acid

EGF	Epidermal Growth Factor
eGFP	Enhanced Green Fluorescent Protein
ELISA	Enzyme Linked Immunosorbent Assay
ES	Embryonic Stem (cell)
FACs	Fluorescent Activated Cytometry
FBS	Fetal Bovine Serum
FKBP	FK506 binding protein
GLP-1	Glucagon-Like Peptide 1
GLP1-R1	Glucagon-Like Peptide 1 Receptor
GlyA	Glycophorin A
GLUT2	Glucose Transporter 2
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GSIS	Glucose Stimulated Insulin Secretion
H_2O_2	Hydrogen Peroxide
HBSS	Hanks Balanced Salt Solution
HDC	Human Pancreatic Ductal Cell
HI	Human Islet
HLA	Histocompatibility Leukocyte Antigen
IBMX	3-Isobutyl-1-methylxanthine
IgG	Immunoglobulin G
ITS	Insulin-Transferrin-Selenium
LG-DMEM	Low Glucose Dulbecco's Modified Eagle's Medium
MgCl	Magnesium Chloride

MODY	Maturity Onset Diabetes of the Young
MOI	Multiplicity of Infection
MSC	Mesenchymal Stem Cell
mPB	Mobilized Peripheral Blood
mTOR	Mammalian Target Of Rapamycin
NeuroD	also known as BETA2
Ngn3	Neurogenin 3
NPI	Neonatal Porcine Islet
PBS	Phosphate Buffered Saline
Pdx1	Pancreatic Duodenal Homeobox-1
RA	Retinoic Acid
RIA	Radioimmunoassay
RO	Retro-Orbital
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SEM	Standard Error of the Mean
Shh	Sonic Hedgehog
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TUNEL	TdT-mediated dUTP nick-end labeling
UCB	Umbilical Cord Blood
VEGF	Vascular Endothelial Growth Factor

CHAPTER I

GENERAL INTRODUCTION

I-A) INTRODUCTION TO DIABETES MELLITUS

Diabetes is a condition that results from physiological perturbations in insulin secretion and/or action that lead to abnormal glycemic control. Currently, more than 2 million Canadians have diabetes, and this number is expected to increase to 3 million by 2010 [1]. There are four major types of diabetes, each with unique pathophysiologies, but all types are characterized by insulin-based changes leading to abnormal blood glucose regulation. These four specific disease states are; type 1 diabetes, type 2 diabetes, maturity onset diabetes of the young (MODY), and gestational diabetes. In terms of prevalence, type 2 diabetes affects 90% of diabetic patients, while type 1 diabetes afflicts the remaining 10%, with only a small fraction (~ 1-2%) of patients having MODY. Gestational diabetes is a transient state of abnormal glycemic control during pregnancy that occurs in 3.5% of pregnancies. This form of diabetes is generally resolved after delivery; however, its occurrence is correlated with an increased risk of developing type 2 diabetes in both mother and child later in life [2].

Type 1 Diabetes Mellitus

Type 1 diabetes (T1D) is an autoimmune disease involving the endocrine pancreas and affects approximately 4.9 million people worldwide [3]. The endocrine pancreas forms approximately 1% of the total pancreatic mass and consists of roughly 1 million micro-organs called islets of Langerhans [4]. Within these islets, there are five cell types: beta, alpha, delta, PP and eta cells. Beta cells are the most abundant cell type of the micro-organ, comprising 60-80% of islet mass. The beta cells secrete insulin, a hormone that is released in a pulsatile rhythm in response to elevations in blood glucose. Insulin plays a crucial role in maintaining blood glucose at physiological levels by facilitating glucose uptake into muscle and adipose tissue and suppressing gluconeogenesis in the liver [4]. As a result of autoimmune beta cell destruction in Type 1 diabetes, insulin is no longer secreted, and exogenous administration of this hormone is required. Without insulin, hyperglycemia results, followed by ketoacidosis, coma, and death [5].

The causative factors of type 1 diabetes are both genetic and environmental, as shown by a 70% concordance rate for diabetes development between genetically identical twins. Both causative factors are multidimensional and are actively being studied. Genetic factors which predispose for this disease include HLA regions on chromosome 6, with 95% of type 1 diabetic Caucasian patients having certain alleles such as DQ2-DR4 and DQ8-DR3 or both [6], while DR2 alleles have been shown to be protective against the disease [7]. The autoimmune reaction in patients with predisposing alleles develops either spontaneously or is triggered by an environmental event that initiates the disease process. Possible environmental factors that are being investigated include early introduction of cow's milk and cereal products, duration of breast feeding, vitamin D levels, and viral infection.

It is known that a longer duration of exclusive breastfeeding is protective against diabetes in both Sweden and Lithuania, countries with high and low type 1 diabetes incidence rates [8], indicating that breastfeeding may confer some protection against disease development. Other protective mechanisms that may be associated with breastfeeding include protection against infections via IgA antibodies in breast milk and enhancement of the infant's own immune response. Increased beta cell proliferation has also been observed in breastfed compared with formula-fed babies [9]. Others have found that 'environmental' factors such as older maternal age [10] and low maternal education [11] increased the incidence of diabetes in offspring. However, the exact variables within these population subsets that confer disease risk are still under investigation. Many associations between viruses and T1D have been reported such as the increased frequency of enterovirus infections in siblings developing T1D compared to non-diabetic siblings [12]. Coxsackievirus B (CVB), rubella virus, rotavirus, and mumps virus are also associated with the induction of T1D [13]. Vitamin D concentrations, which are directly related to duration of sun exposure, have been proposed as an environmental factor that protects against type 1 diabetes development. It is known that there are geographical and seasonal variations in type 1 diabetes diagnosis, with the largest proportion of cases diagnosed during fall and winter and the lowest during the summer [14].

Once the disease process has been initiated by genetic and environmental triggers, beta cell destruction becomes progressively more complete, and exogenous insulin is required by the diabetic patient. Since insulin was discovered in the mid-1920s, traditional treatment for type 1 diabetic patients has consisted of exogenous administration of insulin, first from porcine sources followed by genetically engineered sources [15]. However despite daily insulin injections, diabetes often leads to late complications such as retinopathy, nephropathy, cardiovascular disease, and neuropathy. The Diabetes Control and Complications Trial has shown that tight control of blood glucose through the use of insulin pumps – small packs worn by a diabetic patient that monitor glycemic level multiple times per day and inject insulin in response to hyperglycemia - can delay and diminish the progression of long-term complications [16]. Islet transplantation is another method for achieving tight control of blood glucose in a more physiological way [17]. This procedure involves harvesting pancreatic tissue from cadaveric donors and infusing the isolated islet cell fraction into the portal vein of diabetic patients. The transplanted islets then function from within the liver to regulate glycemic level in a nearly physiological way.

I-B) ISLET TRANSPLANTATION

History

Early islet transplantation was performed by Paul Lacy in 1967 [18], and in 1972 he published work showing that this procedure could successfully reverse diabetes in a rodent model of the disease [19]. Throughout the 1970s and 1980s, significant challenges that prevented the clinical availability of this procedure were investigated [20]. These obstacles included difficulties in reproducing successful islet transplantation in larger animal models and the inability to extract and purify sufficient numbers of islets from the human pancreas. In 1986, an automatic method for human islet isolation was devised, now known as the Ricordi chamber, which enables high numbers of human islets to be isolated and collected [21]. As a result of these improvements in islet isolation, human islet transplantation became a reality. In 1989 the first series of human islet transplantations were performed [22]. Although the transplantation procedure proved successful, these grafts failed largely due to problems with immunosuppression. Throughout the 1990s, immunosuppression continued to be an inhibiting factor for successful islet transplantation with reports of one-third of islet transplants functioning at 1 year post transplantation and with only 10% of transplantations leading to insulin independence [20]. The use of diabetogenic drugs contributed to the limited success of early islet transplantation. Steroids [23] and calcineurin inhibitors [24] were used routinely and these agents impair insulin secretion and action [25]. In 2000, the fate of clinical islet transplantation changed with the introduction of Rapamycin/Sirolimus to the immunosuppressive regime, administered together with Tacrolimus, for islet transplantation [17]. This protocol, now known worldwide as the Edmonton Protocol, restored patients to normal glucose levels, with 100% achieving insulin independence and 80% remaining insulin independent after 1 year.

This protocol has revolutionized how type 1 diabetic patients can be treated, but limitations for widespread islet transplantation still exist due to the need for ongoing immunosuppression and the scarcity of donor islets. Recent reports also suggest that despite effective, non-diabetogenic immunosuppression, islet grafts begin to fail over time [26], with 80% of islet grafts showing c-peptide secretion (a surrogate marker for insulin release) 5 years post transplantation, but with only 7.5% of patients remaining insulin independent. The discrepancy between c-peptide function and insulin independence likely stems from the need for a critical mass of beta cells for the maintenance of blood glycemia at physiological levels [27]. Eisenbarth *et al.* proposed a working model for diabetes development in which beta cell mass is progressively lost over time due to autoimmune destruction of the cells. Once beta cell mass decreases past a threshold, patients require exogenous insulin for proper glycemic regulation. The need for insulin supplementation in islet transplant patients likely results from a similar progression in which beta cell mass is decreasing with time, and when it passes the critical point, proper glycemic control ceases. Possible factors contributing to decreased beta cell mass include limited regeneration of new beta cells to replace dying cells and inhibition of beta cell function or regeneration caused by side effects of the immunosuppressive drugs.

While we know that beta cells are regenerated throughout life, having an average lifespan of 1-3 months [28], the specific cells from which new beta cells are derived are not known. It has been proposed that pancreatic ductal cells undergo a process called neogenesis to replenish the beta cell pool within the pancreas [29]. Pancreatic ductal cells are transplanted together with islets in the islet transplantation procedure [30] and likely enhance the graft function over time. In fact, patients who receive higher numbers of ductal cells show enhanced graft function at 2 years post transplant [30]. It is likely that ductal cells proliferate prior to generating new beta cells in order to maintain sufficient levels of both cells within the pancreas [31]. Sirolimus may inhibit this ductal proliferation due to its mechanism of action [32]. Sirolimus is an effective immunosuppressive agent that functions to inhibit clonal T cell expansion by inhibiting cell cycling from the G1 to the S phase [33]. Since the proteins bound by sirolimus are not unique to T cells, it is possible that this drug exerts its effects on other cell types. Studies have shown that sirolimus inhibits the proliferation of coronary artery smooth muscles [34], hepatic stellate cells [35], and pancreatic cancer cells [36], and it is therefore quite plausible that this drug might be exhibiting similar effects on pancreatic ductal cells and thereby inhibiting longterm islet graft viability.

As the field of islet transplantation moves into its fourth decade, issues at its forefront include investigating possible side effects of immunosuppressive drugs that may contribute to decreased graft longevity and seeking out prolific sources of insulin secreting cells to remedy the shortage of tissue available for widespread transplantation. Potential novel cell sources for islet transplantation include neonatal porcine islets, which have been shown to reverse diabetes in large animal [37-39] and human subjects, and human stem cells.

I-C) STEM CELLS

History

Stem cell research can be traced back to the 1960s when Till and McCulloch, Canadian researchers, began their seminal investigations in experimental hematology that led to their co-discovery of stem cells. By injecting varying amounts of bone marrow cells into mice after the mice had been given lethal irradiation, they observed that those animals receiving higher doses of bone marrow had increased survival rates. From their observations, they developed a definition of stem cells that holds true today: stem cells are self-renewing, able to give rise to differentiated descendants, and capable of extensive proliferation [40].

The first attempts to direct the differentiation of mouse embryonal carcinoma cells were performed in 1978 when Strickland and Mahdavi cultured cells with retinoic acid alone or in combination with dibutyryl cAMP and showed that cells were induced to differentiate into cells that resemble the parietal endoderm [41]. These studies provided the basis for subsequent work attempting to control the differentiation of embryonic stem (ES) cells in vitro. The first mouse ES cells, derived directly from the blastocyst in culture, were reported in 1981 nearly twenty years before this area of research exploded into the public realm [42]. The interest in stem cells increased significantly in the 1970s, paralleling interest in mammalian developmental biology and cell differentiation. The derivation of the first human ES cell lines in 1992 broadened the application of this branch of science to a much wider scientific and general-public audience.

Types

A stem cell is a cell, regardless of its locale of origin, that possesses the ability to self renew, creating new daughter stem cells, and also to yield progeny that have the ability to differentiate into adult cell types. This definition encompasses embryonic, foetal, and adult stem cells. ES cells are derived from the inner mass of the blastocyst, whilst embryonic germ (EG) cells derive from primordial germ cells. In most respects, they are indistinguishable from one another, they are pluripotent, and thus have the capacity to differentiate into all cell types within the body, having germ-line competence, and forming teratomas when injected into immunocompromised mice [42].

Adult stem cells are thought to be cells within the adult that retain considerable differentiation potential and whose purpose is to either repair or replace dead or dying cells throughout an adult's life. There are different types of adult stem cells within the human body, characterized by the physiological locale from which they can be isolated, the cell surface markers that are used to identify such cells, and the differentiation potential of the different adult stem cell types. There have been many reports in the last five to ten years which outline isolation strategies and characteristics of tissue specific adult stem cells from virtually every organ of the body. Some of the most common sources of adult stem cells include blood and bone marrow from which hematopoietic and mesenchymal stem cells can be isolated. Mesenchymal stem cells have also been found in adipose tissue [43] and the pancreas [44], indicating that rather than being restricted to one locale or organ, these adult stem cells may in fact be dispersed throughout the body. Adult stem cells have also been isolated from the skin [45], brain [46], and muscle [47].

A significant challenge for stem cell research has been the identification of adult stem cells. Although these cells generally lack tissue- specific characteristics, to date there is no universal cell surface marker that can be used to detect stem cells from differentiated cells within a given organ. Instead, adult stem cells are currently identified based upon a characteristic panel of cell surface markers that define one stem cell type from another and by the differentiation potential of the stem cell in question.

Adult stem cells generally lack tissue-specific characteristics but can be induced to differentiate into specialized cells with the appropriate stimuli [48]. Physiological stimuli may include cell turnover, wound signals, or disease, all of which might induce the differentiation of endogenous stem cells into progeny capable of replacing injured cells. It was initially thought that although adult stem cells might exist, these cells were only multipotent, or able to differentiate into cells of the organ in which they reside, not pluripotent, or able differentiate into multiple cell types, as had been described with embryonic stem cells. Recent reports, however, suggest that the functional plasticity of somatic tissue (adult) stem cells might be greater than expected. For example, neural cells may transdifferentiate into blood [49] or muscle cells [50] or even be the origin of different germ layer-derivative cells in chimeric mice [51]. Bone marrow cells have also been shown to differentiate into muscle fibers and cardiomyocytes following injury to the respective organs [52], MSCs have been found to express neural phenotype and migrate when placed in damaged brain and spinal cord [53], and hematopoietic stem cells have been shown to differentiate into myogenic cells when injected into ischemic limbs of immunosuppressed mice [54]. Prior to these reports, it was thought that adult stem cells were restricted to differentiating into cell types of their embryological layer; endoderm, ectoderm or mesoderm. But with blood (mesoderm derived) differentiating into neuronal type cells (ectoderm), this longstanding theory was challenged.

Mesenchymal Stem cells

Bone marrow stem cells consisting of hematopoietic and mesenchymal cells have been shown to have considerable potential to differentiate into multiple cell types. Transplanted mouse BM cells can give rise to brain astrocytes in adult mice [55] and can also produce hepatocytes [56], endothelial, and myocardial cells [57] as well as CNS neurons and glial cells [58;59]. The mesenchymal cell fraction within the bone marrow holds much promise for therapeutic use. These cells were first identified by Friedenstein and Petrakova in 1966, who isolated bone-forming progenitor cells from rat marrow [48]. Endogenously, these cells represent 0.001-0.01% of nucleated cells in the marrow and provide the stromal support system for hematopoietic stem cells [60]. While these cells represent a minor fraction of the bone marrow, they can be harvested and expanded in culture with high efficiency. These isolated human cells can now be purchased from companies such as Cambrex Corporation (USA). In culture, MSCs have a fibroblastic morphology and adhere to tissue-culture treated plates where they can be expanded *ex-vivo* for 20-50 population doublings [48;61]. These cells cannot be characterized by a single surface marker but rather by a panel of markers because many markers found on MSCs may also be found on nonstem cells, or a particular marker may only be expressed on a stem cell at a certain stage or under certain conditions [62]. A brief summary of human MSC surface markers compiled by Pittenger *et al.* 2004 is shown below:

Positive
CD13, CD29, CD44,CD49a,b,c,e,f
CD51, CD54, CD58, CD71, CD73,
CD90, CD102, CD105, CD106
CDw119, CD120a, CD120b, CD123,
CD124, CD126, CD127, CD140a,
CD166, P75, TGFb1R, TGFbIIR,
HLA-A,B,C, SSEA-3, SSEA-4, D7

Negative
CD3, CD4, CD6, CD9, CD10,
CD11a, CD14, CD15, CD18,
CD21, CD25, CD31, CD34,
CD36, CD38, CD45, CD49d,
CD50, CD62E,L,S, CD80, CD86,
CD95, CD117(c-kit), CD133, SSEA-1

MSCs express many cell adhesion molecules of potential importance in cell binding and homing interactions [63]. Cellular characterization generally consists of surface marker expression and differentiation potential, with multiple laboratories describing the differentiation of MSCs into bone, cartilage, and fat [48]. Additionally, there have been reports of mesenchymal stem cells differentiating into skeletal muscle [64], and cardiomyocytes [65]. MSCs have also been shown to differentiate across primary germ layers. These mesodermal derived stem cells have been induced to differentiate into ectodermal cells such as neuronal cells [66-68] and into endodermal cells such as hepatocytes [69]. Several studies report the formation of liver cells from transplanted bone marrow in mice [70], rats [71], and humans [69]. In an elegant study, Lagasse *et al.* transplanted bone marrow cells from a mouse ubiquitously labeled with β-galactosidase into lethally irradiated mice with a congenital liver defect [72], resulting in the rescue of the animals from disease onset and death through the restoration of the hematopoietic system and the formation of large β-galatosidase-positive colonies of parenchymal liver cells.

Currently, there is speculation that human blood may be an accessible source of MSCs. Some groups have reported that sources such as mobilized peripheral blood and umbilical cord blood (UCB) contain MSCs [73-76], while others have reported that they do not [77]. Both bone marrow and UCB have been shown to contain nestin positive cells [78]. Nestin is a neuroepithelial marker which is expressed by some progenitor cells of the pancreas in rat and human pancreatic islets [79]. It is possible that nestin positive cells from these extra-pancreatic sources may be able to differentiate into pancreatic beta cells under the proper conditions. Furthermore, UCB cells have been shown to express markers and transcription factors considered important for beta cell differentiation [80], suggesting that cells from this source may be able to differentiate towards the beta cell fate.

Clinical Use of Stem Cells

Until recently, stem cell research has been limited to *in vitro* and *in vivo* animal experimentation. However, with the knowledge and promise gained from this work, there have been several recent reports of stem cell transplantation into human subjects. In an attempt to heal pre-formed fistulas, a small clinical study reported by Garcia-Olmo *et al.* in 2005 investigated the healing potential of autologous adipose derived mesenchymal stem cells on Crohn's fistulas in five patients [81]. Following stem cell injections in the area of the fistulas, they observed a complete healing in six of eight procedures. There is also evidence of the beneficial effects of MSC transplantation following acute myocardial infarction [82]. Following intracoronary injection of bone marrow derived MSCs into 34 patients with acute myocardial infarction, increased wall velocity in the infarcted region and left ventricular ejection fraction were observed in the stem-cell treated group compared to saline-injected controls. In islet transplantation pancreatic ductal cells are also transplanted to the diabetic patient. These ductal cells are hypothesized to be adult stem cells within the pancreas that undergo neogenesis to form new islets, replacing dead or dying cells within the normal pancreas [29;83-85]. A correlation exists between the number of ductal stem cells that a patient receives and their graft function at two years post-transplant, indicating that ductal cells within islet grafts may be contributing to sustained pools of islets and therefore preserving graft function over time [30].

I-D) BETA CELL DIFFERENTIATION

The essence of stem cell research is to synthetically direct and recapitulate the differentiation events that occur during normal organ development. As such, a thorough understanding of the developmental biology of the organ of interest is required. Our goal is to generate insulin-secreting beta-cells from adult stem cell sources. Thus, we will briefly review what is currently known about beta cell development and review strategies that have been employed to reproduce this development experimentally.

Transcription factors

Beta Cell Development

During the gastrulation process of embryonic development, three germ layers are formed: the ectoderm, endoderm and mesoderm. Pancreatic endocrine cells derive as part of the primitive gut from the endoderm germ layer. Around embryonic day e9 in mice, the pancreas buds into two parts - the dorsal and ventral - from the foregut. The ventral endoderm of the foregut gives origin to the thyroid gland, lungs, and liver, as well as the ventral part of the pancreas and the dorsal endoderm, the origin of intestine and the dorsal part of the pancreas [3]. The dorsal endoderm is in contact with the notochord, while the ventral endoderm is close to the cardiac mesoderm. As development progresses, the two buds of the pancreas fuse together, with the developmental fate of the cells within these buds being regulated by extracellular signals from neighboring cells which lead to the expression of tissue- and cell type-specific patterns of transcription factors.

The repertoire of transcription factors that drive differentiation of endodermal cells to beta cells has been characterized and reviewed [31;86-88]. The initiating factor for this directed differentiation is the repression of Sonic Hedgehog (Shh) by the notochord, which in turn promotes pancreatic duodenal homeobox-1 (Pdx1) expression in adjacent pancreatic endoderm. Pdx1 is a homeobox transcription factor that is highly conserved across species and is expressed mainly in the endocrine pancreas and in subsets of the enteroendocrine cells of the duodenum and pyloric stomach [89]. Gene disruption of this factor abrogates normal pancreatic development in mice, resulting in selective loss of the enteroendocrine cell population and mice homozygous for a targeted mutation in the Pdx1 gene are apancreatic [90-92]. In humans, inactivation of Pdx1 is associated with pancreatic agenesis [93]. Pdx1 is a key factor in pancreatic development in that it regulates beta cell differentiation and insulin gene expression and maintains normal beta cell function by regulating several beta cell related genes [3;91]. Pdx1 gene expression persists throughout the early stages of pancreatic development, while during the later stages of development by e18.5, the expression of Pdx1 becomes mostly restricted to the mature beta cells of the endocrine pancreas [87].

Important transcription factors expressed by the developing beta cell downstream of Pdx1 include ngn3, NeuroD/Beta2, Pax4, Pax6, Nkx6.1 and Nkx2.2. Between e14 and e17, pancreatic endocrine cells are derived from a subset of duct cells [94] transiently expressing the transcription factor ngn3 [95]. From these ngn3 expressing cells, transient Pax4 expression specifies the beta cell phenotype, and differentiated beta cells eventually express high levels of Pdx1, Nkx6.1, Nkx2.2 and Pax6 transcription factors [96]. Ngn3 is required for the development of all endocrine cells of the pancreas and is a marker of islet precursor cells [97]. Expression of this factor starts on e9-e9.5, peaks on e15.5 when there is a major wave of endocrine cell genesis, and is greatly diminished at birth, with little or no detection of ngn3 in the adult pancreas [87]. Overexpression of ngn3 in the developing pancreas results in accelerated differentiation of endocrine progenitor cells, while mice carrying a null mutation for this factor fail to generate any endocrine cells or endocrine precursors during development [97]. Ngn3 directs differentiation of pancreatic precursor cells towards endocrine lineages, while NeuroD, a transcription factor activated by ngn3, is required for islet growth and proliferation and for insulin activation in the mature beta cell [87;98]. NeuroD, also known as BETA2, is expressed in pancreatic and intestinal endocrine cells and in terminally differentiated neurons [87;91]. Islets of mice with a targeted disruption of this gene have islets that are dysmorphic with irregular aggregates and reduced numbers of beta cells [99].

The Pax and Nkx genes are also important for normal beta cell development and function. Pax4 and Pax6 are genes expressed by the central nervous system and the endocrine pancreas [100-102]. Pax4 null mice have no beta and delta cells but show increased alpha cells [100], while mice with mutations in the Pax6 gene have abnormal organization of islets with decreased beta, alpha, delta and PP cells [103]. The Nkx genes Nkx6.1 and Nkx2.2 are also regulators of pancreatic endocrine cell differentiation. Nkx2.2 is expressed in the alpha, beta, and PP cells of islets, while Nkx6.1 is expressed primarily in the beta cells of adult islets [87;104]. The pancreas of Nkx2.2 and Nkx6.1 knockout mice have no insulin producing cells and diminished glucagon producing cells, while the exocrine pancreas remains histologically normal. Cells from these mice have precursor cells that express endocrine pancreas specific proteins such as synaptophysin and amyloid polypeptide but are hormone negative [105].

Experimental differentiation

Using the knowledge that there are key transcription factors which guide the development of the endocrine pancreas, researchers have done much experimental work that involves transducing stem cells or somatic cells with viral vectors to introduce these key transcription factors intracellularly with the aim of inducing differentiation towards the endocrine pancreas cell fate. Using a tissue-specific promoter in a Xenopus model, Horb *et al.* overexpressed an activated from of the Xenopus homolog of PDX-1 in the liver, resulting in a conversion to pancreatic tissue containing both exocrine and endocrine cell types [106]. In a similar study, Zalzman *et al.* induced immortalized human fetal liver progenitor cells to differentiate into insulin-producing cells by viral transfection of PDX-1 [107]. Kojima *et al.* have demonstrated the induction of islet neogenesis using adenovirus to deliver a combination of NeuroD and betacellulin to hepatic cells [108]. Kaneto *et al.* found that when PDX-1 was ectopically induced in the liver, the insulin2 gene was expressed. In addition, larger amounts of insulin2 gene expression were induced in the liver through the overexpression of PDX1 as well as NeuroD or ngn3. They reported that 3 days after adenovirus injection, blood glucose levels were moderately decreased by PDX1 alone and more significantly decreased by overexpression of PDX1 as well as NeuroD or ngn3 [91]. Experimental evidence also suggests that human cells can be induced to differentiate towards the pancreatic endocrine cells fate following viral transduction. Successful expression of insulin was induced in adult human pancreatic ductal cells following ectopic adenoviral expression of ngn3 [109]. Infection resulted in gene and/or protein expression of NeuroD, Pax4, Nkx2.2, Pax6, and Nkx6.1 as well as a 15-fold increase in the percentage of synaptophysin and insulin positive cells relative to control cells. Unregulated insulin secretion has also been induced in human MSCs following retroviral infection with human preproinsulin cDNA [110]. These cells, however, did not exhibit beta cell specific functions such as glucose stimulated insulin secretion – functions that would presumably result from inducing the differentiation of a beta cell by administering genes expressed early in beta cell development rather than simply inducing a cell to secrete insulin.

Soluble factors

Beta Cell Development

In concert with activation of the transcription factors discussed above, there are many soluble factors present within the developmental environment of the embryonic pancreas that function to direct cellular differentiation. Chicken and mouse studies have shown that fibroblast growth factor 2 (FGF2) acts on specific receptors to repress Shh expression, allowing for upregulation of Pdx1 [3]. This growth factor has also been shown to stimulate the proliferation of pancreatic progenitor cells [111]. Pancreas development is also known to be influenced by signals from the mesodermal germ layer (the dorsal aorta, notochord, and pancreatic mesenchyme) [112]. The pancreatic bud growth is regulated by factors including bone morphogenic proteins (BMP), FGFs, retinoic acid (RA) and other soluble factors. Mesenchymal factors such as follistatin repress endocrine cells and induce exocrine differentiation [113]. It is also known that activation of retinoic acid receptors during gastrulation specifies the location of Pdx1 expression along the anterior-posterior axis of the endoderm in zebrafish [114].

Experimental differentiation

Many soluble factors have been investigated experimentally regarding their ability to enhance beta cell differentiation. Glucose, GLP-1/exendin 4, activin A, nicotinamide, EGF, and FGF have all been shown to enhance pancreatic beta cell development and function. Glucose is known to activate Pdx1, resulting in activation of cytoplasmic Pdx1 and translocation to the nucleus [115]. Glucose infusion in rats also results in 50% increases in beta cell numbers after being administered for 24 hours [116].

The gastrointestinal incretin hormone glucagon-like peptide-1 (GLP-1) and its analog exendin 4 have been found to induce differentiation of ductal and acinar cells to functional beta cell phenotype [117;118]. Experimental evidence also exists showing that activin A and betacellulin can convert AR42J acinar cells into insulin producing cells [119] and can reduce plasma glucose when administered to streptozotocin-induced diabetic rats [120]. Nicotinamide is known to enhance insulin secretion and beta cell differentiation in fetal porcine islet like clusters [121], while *in vivo*, the administration of gastrin and EGF has been shown to restore glycemic control in alloxan-induced diabetic mice [122]. Despite these successes, there is still no defined protocol for using these betagenic factors which yields successful, consistent differentiation or regeneration of pancreatic beta cells.

The challenge for the administration of individual growth factors for beta cell differentiation is that during embryogenesis, the expression of these factors (as well as many undefined factors) is transiently expressed in doses and combinations that are difficult to ascertain ex vivo. While we have marginal knowledge about the physiological dose and combination of growth factors required to yield optimal beta cell differentiation and regeneration ex vivo, it is well known that these events do occur physiologically following pancreatectomy in mice [31;123-125] and in response to pregnancy [126] and obesity [127] in humans. Based on this knowledge, a different approach to achieve beta cell differentiation is to expose progenitor or stem cells to this regenerative environment. This has been accomplished and shown to be effective by either inducing pancreatic injury [128;129] to initiate regeneration or adding pancreatic extract to cultured cells [130;131].

I-E) STEM CELLS AND BETA CELL DIFFERENTIATION

Multiple approaches are currently being explored to generate insulin secreting cells in vitro, either by using various potential beta-cell precursor cells such as pancreatic ductal cells or by using adult stem cells such as mesenchymal stem cells. Some promising results in this area have already been obtained using ES cells [132-134]. However, the potential use of ES cells for the treatment of diseases in humans is complicated by practical and ethical issues. This work will focus on the use of adult stem cells for the treatment of diabetes. We will review potential cell sources and describe different approaches for stem cell differentiation.

Potential Adult Cell Sources

Adult Progenitor Cells

Pancreatic ductal cells, identified by cytokeratin (CK) intermediate filament proteins, are believed to be beta cell progenitors within the pancreas, contributing via neogenesis to the generation of new beta cells. Evidence leading to this belief includes the observation of 'transitional cells' expressing both insulin and CK19 in adult human pancreatic sections [64] and the appearance of individual beta cells and intact islets in close contact with ductal epithelium [63, 64]. It is also known that in early pancreatic development branching morphogenesis of the ductal structures occurs [61, 62] and cells within these structures eventually lose CK expression and develop into both the endocrine and exocrine compartments of the pancreas [61]. Our group has recently shown that islet recipients who receive greater numbers of ductal cells within islet grafts have better graft function at 2 years post transplant as measured by AIRg [30]. These observations indicate that ductal cells within the graft retain the ability to differentiate into beta cells, which contribute to the sustained graft function over time.

Experimental evidence also supports the theory that islet neogenesis in the mature pancreas occurs from ductal cells. Viral transduction of ductal cells with ngn3, a transcription factor expressed during pancreatic development, has been shown to initiate differentiation towards the beta-cell phenotype [109]. Ductal-derived 'islet buds' have also been observed in both human and rat cultures, with the cells expressing islet hormones [65,66].

Other cell sources that may contribute to beta-cell turnover in the adult pancreas include beta-cells themselves and pancreatic acinar cells. Using a genetic lineage tracing approach, Dor *et al.* have reported that adult pancreatic beta cells are generated by self-duplication rather than from differentiation of pancreatic duct cells or stem cells [135]. It has also been shown that transdifferentiation of acinar cells into duct-like cells may occur, with the transient duct-like cells progressively differentiating into endocrine cells [73, 96-99].

Mesenchymal Stem Cells

While pancreatic progenitor cells have been shown to be the likely cell source which replenishes beta-cells in vivo, the use of these cells and their differentiation products for islet transplantation rely upon cadaveric organ donation – a significant limiting factor for obtaining unlimited tissue supply. While beta cells, ductal cells, acinar cells, and liver cells may possess the inherent capability of differentiating into new beta cells, these sources are not practical tissue sources for islet transplantation because they cannot be harvested without incident to the donor. Source organs such as blood and bone marrow, however, can be harvested with ease, and stem cells could potentially be harvested from the patient, thereby circumventing graft rejection issues and the use of immunosuppressive drugs.

Experimental evidence shows that MSCs from extra-pancreatic sources within the body such as bone marrow and blood may be capable of undergoing differentiation towards the beta-cell fate. Evidence leading to this belief comes mainly from work with rodent cells. However, recent work has shown that human MSCs also possess similar differentiation capacity.

The first reports of MSC differentiation towards the pancreatic beta cell fate were published in 2003. Since this date, there have been conflicting reports on this topic, most likely stemming from inconsistencies between protocols used by different groups. Early work in this area involved transplantation of whole bone marrow into diabetic mice. Experimental evidence demonstrated that bone marrow-derived stem cells can initiate pancreatic regeneration when pancreatic damage occurs prior to the transplantation of cells. With the use of this damage model, hyperglycemic mice have been shown to have reduced blood glucose levels within 7 days of cellular infusion [123] and also have bone marrow derived insulin-producing cells within the pancreatic tissue [136]. It is also known that bone-marrow derived cells persist for up to six weeks after transplantation and can differentiate into insulin positive cells within the pancreas, expressing beta-cell specific transcription factors and showing glucoresponsive insulin secretion [137]. Conflicting reports show that transplanted bone marrow cells migrate to the pancreas following intravenous infusion but that these cells do not express insulin [138]. Although pancreatic damage was used in this work to attract cells to the damaged organ, the damage was inflicted 5 weeks following bone-marrow transplantation. This long interval between cellular transplantation and organ damage is likely the cause of the lack of evidence of transdifferentiation being observed. There have also been recent reports [139] that have attempted to reproduce earlier results [137] without success. This recent report found CD45 positive bone marrow cells within the pancreas and concluded that the infused cells had adopted a hematopoietic cell fate.

When whole bone marrow (containing differentiated hematopoietic cells, hematopoietic stem cells, and mesenchymal stem cells) is infused, problems arise with defining the cell fraction within this heterogeneous mixture that has the potential to give rise to insulin positive cells. In response to this issue, experimental work in subsequent studies has characterized and defined the fraction of bone marrow cells that are used for differentiation towards the beta cell fate. Many consistent reports show that the mesenchymal stem cell fraction from rodent and human bone marrow can be induced to differentiate towards the pancreatic beta cell fate.

Evidence leading to this belief comes from work showing that rat MSCs can be induced to express and secrete insulin using a differentiation protocol consisting of DMSO, high glucose, and collagen-coated culture surfaces [140]. These cells formed small spheroid clusters containing insulin, large vacuoles, and secretory vesicles, and the cells also had microvilli on their surfaces, features that are characteristic of beta cells. When differentiated clusters were transplanted beneath the kidney capsule of diabetic mice, the blood glucose began to normalize within 3 days and hyperglycemia recurred upon nephrectomy of the graft bearing kidney. It is also known that when rat MSCs are cultured with a progressive differentiation protocol consisting of low glucose, beta-mercaptoethanol, nicotinamide, and high glucose, the morphology of cells changes from fibroblast-like cells to round or oval cells containing granules and expressing insulin mRNA [141]. Cells are only partially differentiated, however, as they do not express insulin protein with this protocol. Similar approaches involving long-term culture in low or high glucose followed by sequential exposure to low glucose, nicotinamide and exendin 4 also report cell cluster formation [142]. In this work, clusters were positive for insulin and c-peptide and expressed the GLP-1 receptor gene, indicating that this approach for beta cell differentiation of rodent MSCs is successful and reproducible. Rodent MSCs can also be induced to differentiate towards the beta cell fate following in vitro culture with rat pancreatic extract derived from partially pancreatectomized rats [143]. Using this methodology, cells changed morphologically to yield islet-like clusters and expressed mRNA for insulin, glucagon, pancreatic polypeptide, somatostatin, GLUT-2, Pax4, and Nkx6.1. Glucose-sensitive insulin secretion was also observed, although cells produced less insulin than normal pancreatic islet cells.

Recent reports show that human MSCs are also capable of differentiating toward the pancreatic beta-cell fate. Following 3 days of culture with media supplementation, human adipose derived MSCs were shown to upregulate expression of the key pancreatic genes Pdx1, ngn3, and Isl1 as well as genes for insulin, somatostatin and glucagon [144]. C-peptide positive cells were also detected, and there was cellular release of somatostatin. While this study did not report insulin secretion from differentiated MSCs, evidence also exists showing that these human cells can be induced to secrete human insulin stably following retroviral infection with the human insulin gene [145] and human preproinsulin cDNA [110]. hMSCs transfected with the insulin gene expressed the insulin gene transcript and secreted insulin into culture for > 3 weeks, indicating that MSCs possess the cellular machinery necessary for transcription, translation, and cleavage of the insulin protein. The evidence that gene-transfected hMSCs can secrete insulin is promising; however, in order to address the shortage of insulin secreting cells for islet transplantation, regulated glucose stimulated insulin secretion is required. Should cells which secrete a basal amount of insulin be transplanted to diabetic patients, hypoglycemia would result.

One approach to achieve glucose-sensitive insulin secretion from MSCs is to transfect the cells with key genes expressed by the developing pancreas in order to stimulate the cells to differentiate into beta cells possessing the cellular proteins necessary for regulated insulin secretion. Moriscot *et al.* (2005) explored this approach by using a combination of infection with adenoviruses coding for mouse Ipf1/Pdx1, HlxB9 and FoxA2 with either islet conditioned media or islet co-culture [146] and reported that MSCs demonstrated upregulation of selective pancreatic transcription factors and insulin gene expression. While these results show promise, further investigative work is needed in this field to confirm that human MSCs have the same capacity for beta cell differentiation that has been seen with rodent MSCs such as beta cell protein expression, glucose-stimulated insulin secretion and in vivo glycemic regulation by differentiated MSCs.

I-F) SUMMARY

In order to provide widespread availability of islet transplantation for diabetic patients the issue of tissue shortage will likely be resolved by the use of adult stem cells including human MSCs, pancreatic ductal cells and porcine islets. There have been many scientific advances in the last four years which demonstrate the potential of mesodermal derived stem cells such as MSCs to cross embryological lineage boundaries and differentiate into endodermal derived cells with pancreatic beta cell characteristics. Key factors that are known to be expressed during beta cell development and that have been shown to be effective at inducing this MSC differentiation include Pdx1, ngn3 and NeuroD. Soluble factors that enhance beta cell differentiation and function include glucose, exendin 4, activin A, nicotinamide and undefined factors secreted by the regenerating pancreas. Work using these approaches to direct MSC differentiation in rodent cells has shown that these cells have the potential to differentiate towards the beta cell fate showing cell cluster formation and insulin expression. Recent reports also show that human MSCs appear to have similar differentiation capabilities, with reports of c-peptide positive cells being derived from MSC cultures and the upregulation of pancreatic transcription factors and insulin gene expression in differentiated cells.

In summary, much work has been done investigating MSC to beta cell differentiation in rodents while few studies have reported such differentiation with human MSCs. Strategies that have been shown to be effective using rodent cells must be demonstrated with human cells and new strategies developed which yield differentiation into functional beta cells which are capable of glucose sensitive insulin secretion. Further work is also needed to explore adult cell sources from which clinically accessible MSCs can be derived. Evidence exists that these cells may reside in blood, however a thorough characterization of isolation procedures and resulting cell populations is needed. Finally, if allogeneic adult stem cells or xenogeneic islets are to be transplanted into diabetic patients immunosuppression will be needed. As such it will be important to determine the effects of these drugs on stem cell function.

I-G) OBJECTIVES AND GENERAL OUTLINE OF THESIS

The work presented here contributes novel information to the body of scientific knowledge surrounding human adult stem cells and beta cell differentiation.

Specific objectives include:

1. ***To determine the effect of ngn3 and Pdx1 on the differentiation of human mesenchymal stem cells into insulin secreting cells.***
2. ***To characterize the effect of time and sequenced administration of pancreatic transcription factors on the differentiation of human mesenchymal stem cells.***
3. ***To determine the potential for the isolation of MSCs from mobilized peripheral blood and umbilical cord blood***
4. ***To investigate the effect of Sirolimus on the proliferation and function of pancreatic islets and ductal cells.***

Following thorough investigation and discussion of each of these objectives this thesis will conclude with a discussion of the work as a whole, its broad implications and future directions to be explored within the field of adult stem cells and diabetes.

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CHAPTER II

THE EFFECT OF NGN3 AND PDX1 ON THE DIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS INTO INSULIN SECRETING CELLS

II-A) INTRODUCTION

Several research studies suggest that mesenchymal stem cells (MSCs) of the bone marrow are able to differentiate into endodermal cells, such as pancreatic endocrine cells and hepatocytes [1-5]. Evidence of this differentiation process is still being accrued, with the specific mechanisms of gene transcription and cell signaling remaining largely unknown. If a reliable method for MSC differentiation into pancreatic beta cells is established, this cell source could address the shortage of human donor tissue available for widespread islet transplantation [6]. To address this challenge we have examined the possibility of inducing beta cell differentiation from MSCs, by engineering expression of key embryonic transcription factors within the MSCs and culturing these cells with soluble factors that are instrumental for beta cell differentiation.

Pdx1 and ngn3 are key transcription factors expressed during the developmental cascade of pancreatic beta cells development. Cells expressing Pdx1 give rise to all pancreatic tissues: exocrine, endocrine and duct [7]. This transcription factor is essential for pancreas development as Pdx1 deficient mice do not develop a pancreas [8;9]. Ngn3 is expressed downstream of Pdx1, and is restricted to those pancreatic cells that will progressively mature into pancreatic endocrine cells [10]. Mice with a targeted disruption of ngn3 have no endocrine cells [11], demonstrating the importance of this factor for normal pancreatic endocrine development.

Ectopic expression of ngn3 and Pdx1 can induce differentiation towards the beta cell fate in many cell types [12-14]. Research evidence shows that adenovirus-mediated ngn3 transduction induces adult human pancreatic duct cells to express insulin [14], and that co-expression of both Pdx1 and ngn3 induces insulin gene expression in mouse liver cells [12]. Ectopic transduction of Pdx1 can also lead to insulin gene expression in human bone marrow MSCs [4].

High glucose [1;13;15], nicotinamide [16], and exendin-4 [17-19] are contributing factors for beta cell differentiation. High glucose likely provides a physiological stimulus which initiates beta cell development to yield increased insulin secretion and resulting in enhanced glycemic regulation. Nicotinamide is a second factor known to enhance beta-cell differentiation, while exendin 4, a GLP-R1 agonist currently used clinically in the US as a treatment option for T2D patients, increases beta cell proliferation and glucose stimulated insulin secretion. A combination of these three factors have been shown to be effective for inducing murine bone marrow differentiation into functional insulin secreting cells [20]. While evidence is accumulating showing that the differentiation of rodent MSCs into insulin secreting cells is possible [1;2;20;27], evidence for differentiation of human cells towards the same fate remains sparse [4]. To investigate this gap, the purpose of the current study was to direct the differentiation of human bone marrow MSCs into insulin secreting cells by providing physiological stimuli. To achieve this goal, we combined multiple differentiation strategies by ectopically expressing ngn3 and Pdx1 alone or simultaneously, in combination with soluble differentiation factors, to assess the role of these components in initiating pancreatic differentiation and insulin secretion in human bone marrow MSCs.

II-B) METHODS

Cell Culture

Cells were obtained from Cambrex Bioscience Walkersville Inc (Walkersville, MD, USA). Human MSCs, obtained from normal human bone marrow of a 32-year-old Caucasian male and a 19-year-old Asian male, were used for the differentiation protocols. MSCs were plated at a density of 5000 cells/cm^2 and cultured with Mesencult growth medium with Stimulatory Supplements (Stem Cell Technologies, Vancouver, BC, Canada), at a volume of 0.5mL media/cm^2 and used between passages 1 and 7. Media was replaced every 2-3 days and cells were passaged at 70-80% confluence.

Differentiation Protocols

To characterize the mesodermal differentiation potential of the cultured MSCs, osteogenic and adipogenic differentiation protocols were conducted prior to experimental pancreatic differentiation strategies. For osteogenic differentiation, MSCs were grown to 80% confluence and treated with Osteogenic-Selective Medium (Stem Cell Technologies, Vancouver, Canada) according to the manufacturer's protocol. The medium was replaced every 3-4 days for 20-31 days, at which point cell cultures were stained for markers of osteogenesis. To achieve adipogenic differentiation, cell cultures were grown to 80% confluence and treated with Adipogenic-Selective Medium (Stem Cell Technologies, Vancouver, Canada). The medium was replaced every 3-4 days for 14-20 days, at which point cultures were stained for markers of adipogenesis.

Adenoviral transduction

Human bone marrow derived MSCs were transduced with a replication-deficient adenovirus, containing a vector with the genes for enhanced green fluorescent protein (eGFP) and for murine ngn3 or rat Pdx1, driven by separate CMV promoters (gift from Dr. Harry Heimberg, Free University of Brussels, Belgium), using a modification of a previously described protocol [14].

Briefly, adherent MSCs (70-80% confluent) were trypsinized with 0.25% trypsin-EDTA and cell number was determined using a hemocytometer. Cells were seeded at a density of 25000 cells/cm^2 into tissue culture treated plates with 59μl LG-DMEM/cm^2. Cultures were infected at a multiplicity of infection (MOI) of 50 with adenovirus for 4 hours at 37°C, and subsequently washed two times with HBSS to remove virus particles and re-cultured with control (low glucose Dulbecco's modified eagle's medium, LG-DMEM, Invitrogen) or supplemented media (supplementation protocol follows). Preliminary infection efficiency was judged at 48 hours post-infection by visualization of GFP expression on a Zeiss Axiovert 25 Inverted fluorescent microscope (Carl Zeiss Canada, ON, Canada) with a Coolsnap camera and IP lab software (Scanalytics inc, Fairfax, USA). Cultures infected with the adenoviral vector containing ngn3 were cultured for up to 20 days in LG-DMEM and cultures assessed for morphology, gene expression by RT-PCR, and protein expression by immunohistochemical staining, at 48 hours, 10 days, 15 days and 20 days post infection. Cell cultures infected with either ngn3 alone or dually infected with ngn3 and Pdx1were cultured with and without a multi-step supplementation protocol (detailed below). These cultures were characterized for morphology throughout a 20 day culture period. Gene expression, protein expression, and neutral red dye uptake were determined at day 20 of culture.

Multi-step supplementation protocol

In conjunction with adenoviral infection, cell cultures were treated with a four-step differentiation protocol similar to what we have described elsewhere [21]. Briefly, cell cultures, at 80% confluence, were first treated for 3 days with 1% DMSO in LG-DMEM, followed by a 14 day culture with high glucose (HG) DMEM (Invitrogen) supplemented with 1% FBS (Sigma). Thirdly, cells were cultured with LG-DMEM, supplemented with 1% FBS, and 10 mM nicotinamide for 7 days. The final step involved supplementation of LG-DMEM medium with 1% FBS, 10 mM nicotinamide and 10 nM exendin 4 (Sigma) for 7 days. Throughout the differentiation period all media formulations contained 100U/mL penicillin and 100ug/mL of streptomycin and complete media was replaced every 2-3 days.

Cell Characterization

Flow Cytometry and Cell Morphology

Prior to the initiation of experimental differentiation protocols, MSCs were harvested and characterized by flow cytometry using antibodies (dilution) for CD13 (3/50), CD29 (3/50), CD45 (3/50), CD90 (1/50), CD34 (2/5) (BD Biosciences, Mississauga, ON, Canada), CD44 (1/10) (Santa Cruz Biotechnology, CA, USA), CD49b (1/10) (Sanquin, Amsterdam, The Netherlands), CD117/c-kit (1/5), CD49d (1/50) (Chemicon International, Temecula, CA, USA), and CD54 (1/50) (Caltag Laboratories, Burlingame, CA, USA). Cells were fixed with cold 1% formaldehyde (BDH Laboratory Supplies), washed once with PBS, and stained with primary antibodies prior to analysis with a FACS Calibur machine (BD Biosciences, Mississauga, Canada) and Cell Quest Pro software and compared to the isotype control.

Cell morphology was characterized throughout the course of the differentiation period by phase contrast microscopy using a Zeiss Axiovert 25 inverted fluorescent microscope (Carl Zeiss Canada, ON, Canada) with a Coolsnap camera and IP lab software (Scanalytics inc., Fairfax, USA). Throughout the experimental period MSCs began to form cell clusters. To determine if these clusters consisted of live or dead cells, cultures were stained with a neutral red solution by the addition of 60 µl of 2.5 mg/ml (final concentration 0.01%, wt/vol) stock neutral red solution (Fisher Scientific, Edmonton, Canada) to each well for 30 min and the number of neutral red-positive (live) and red-negative (dead) cells was determined.

Immunohistochemistry

For the first arm of this study, cells were treated in one of three ways – untreated controls, infected with adenovirus containing GFP alone, or infected with adenovirus containing vectors for both GFP and ngn3. Cells from each treatment were collected for immunohistochemical analysis at 48 hours, 10 days and 20 days post infection. Adherent cells were suspended using a cell scraper and were then allowed to adhere to histobond slides and were fixed in Bouin's fixative for 12 min before storage at 4°C in 70% ethanol. For the second arm of the study, cells were grown in chamber slides and adherent cells were fixed with Bouin's fixative for 12 minutes and similarly stored at 4°C in 70% ethanol. Immunostaining was performed using the ABC-DAB (Avidin-Biotin Complex visualized with 3,3 diaminobenzidine) method. Cells were quenched with a 20%

H_2O_2/80% methanol solution, and microwave antigen retrieval was performed for 15 min on high power (Sanyo,1260W) in 800 ml 10 mM sodium citrate solution (pH 6.0) for ngn3 staining and in Tris EDTA (pH 6.0) for Pdx1 staining. Blocking was performed with 20% normal goat serum (Fisher) for 15 min. Primary antibody concentrations were as follows: 1:200 polyclonal rabbit anti-human ngn3 (gift from Dr Michael German, University of California, San Francisco, USA); 1:1000 guineapig anti-porcine GFP (Dako, Denmark); 1:2500 rabbit anti-human Pdx1 (Chemicon Internation, CA). Primary antibody incubations were for 30 min at room temperature, with the exception of Pdx1 which involved an overnight incubation at 4 °C. Following this incubation, slides were washed (three times) with PBS before the addition of the secondary antibody. For ngn3 and Pdx1 staining, the biotinylated secondary antibody was goat anti-rabbit and for GFP staining the biotinylated secondary antibody was goat anti-mouse. All secondary antibodies were obtained from Vector Laboratories (Burlingame, CA, USA) and used at a concentration of 1:200 for 20 min at room temperature. ABC complex (Vector) incubation time was 40 min at room temperature and visualization was with the chromagen diaminobenzadine (Biogenex, San Ramon, CA, USA) for 5 min. Slides were coverslipped with entellan (EM Science, Gibbstown, NJ, USA) and images were captured on a Zeiss Axioskop II fluorescent microscope with a Coolsnap camera and IP lab software (Scanalytics Inc., Fairfax, VA, USA). Single cell counts were performed on a minimum of 500 cells per sample and percentages were calculated as the number of positive cells per 500.

RT-PCR

Cell samples were pelleted, snap frozen in liquid nitrogen, and stored at -80°C prior to molecular analysis. All samples were subsequently thawed and RNA was extracted using Qiagen RNeasy mini kit (Qiagen Inc. Missassauga, ON, Canada). cDNA was constructed from 1 µg RNA with 10 units (200U/µl) Superscript reverse transcriptase in 1X buffer containing 0.01M DTT, 0.5mM dNTPs, and 0.02 µg/µl oligo dT15. For each sample, 2 µl of cDNA was used per 30 µL reaction along with 1X PCR buffer, 2 mM MgCl, 0.2 mM dNTPs, 1 unit Platinum taq polymerase (5U/µl), and 0.5 µM of each primer pair. Primer pairs employed included human insulin, ngn3, Pdx1, Synaptophysin, Glucagon, Nkx 2.2, Nkx 6.1, Pax4, Pax6, NeuroD, β2 microglobulin, mouse ngn3 and rat Pdx1. Primer sequences are described elsewhere [14;21;22]. All chemicals/reagents were from Gibco/Invitrogen. For PCR reactions, 35 cycles were performed, with 30 s denaturation at 94 °C, 30 s annealing at 58 °C and 30 s extension at 72 °C. Final polymerization was at 72 °C for 10 min followed by a 4 °C hold. Products were separated on a 2% agarose gel and images were captured with Alpha Digidoc software (Perkin-Elmer, Boston, MA, USA). All PCR reactions included a positive control of human islet cDNA and a negative control of Sigma water (Sigma, Oakville, ON, Canada) in place of cDNA.

Data Analysis

Values are expressed as mean ± SEM of n independent observations. Statistical significance was calculated with a two-tailed unpaired Student's t test, or a one-way ANOVA and Scheffe's test in the case of multiple comparisons, using SPSS 14 software (SPSS Inc., Chicago, Illinois). All significance levels were set at $P < 0{\cdot}05$.

II-C) RESULTS

Characterization of hMSCs and demonstration of pluripotency

A thorough analysis of the hMSCs used in this study was carried out prior to the initiation of the experimental differentiation protocols. From this analysis, we definitively showed (Figure 2-1 a and b) that the cells used in our differentiation protocols, were MSCs as defined by Pittenger *et al.* [23]. Cells were positive for the characteristic hMSC surface receptors CD13, CD29, CD44, and CD90 and were negative for the hematopoietic markers CD49b, CD54, CD34, CD45, CD49d, and CD117. We also demonstrated the mesodermal functional pluripotency of these cells by adipogenic and osteogenic differentiation as demonstrated by oil-red-O staining for lipid droplets (Figure 2-1c) and Von Kossa and trichrome staining for mineralization and collagen deposition respectively (Figure 2-1 d and e).

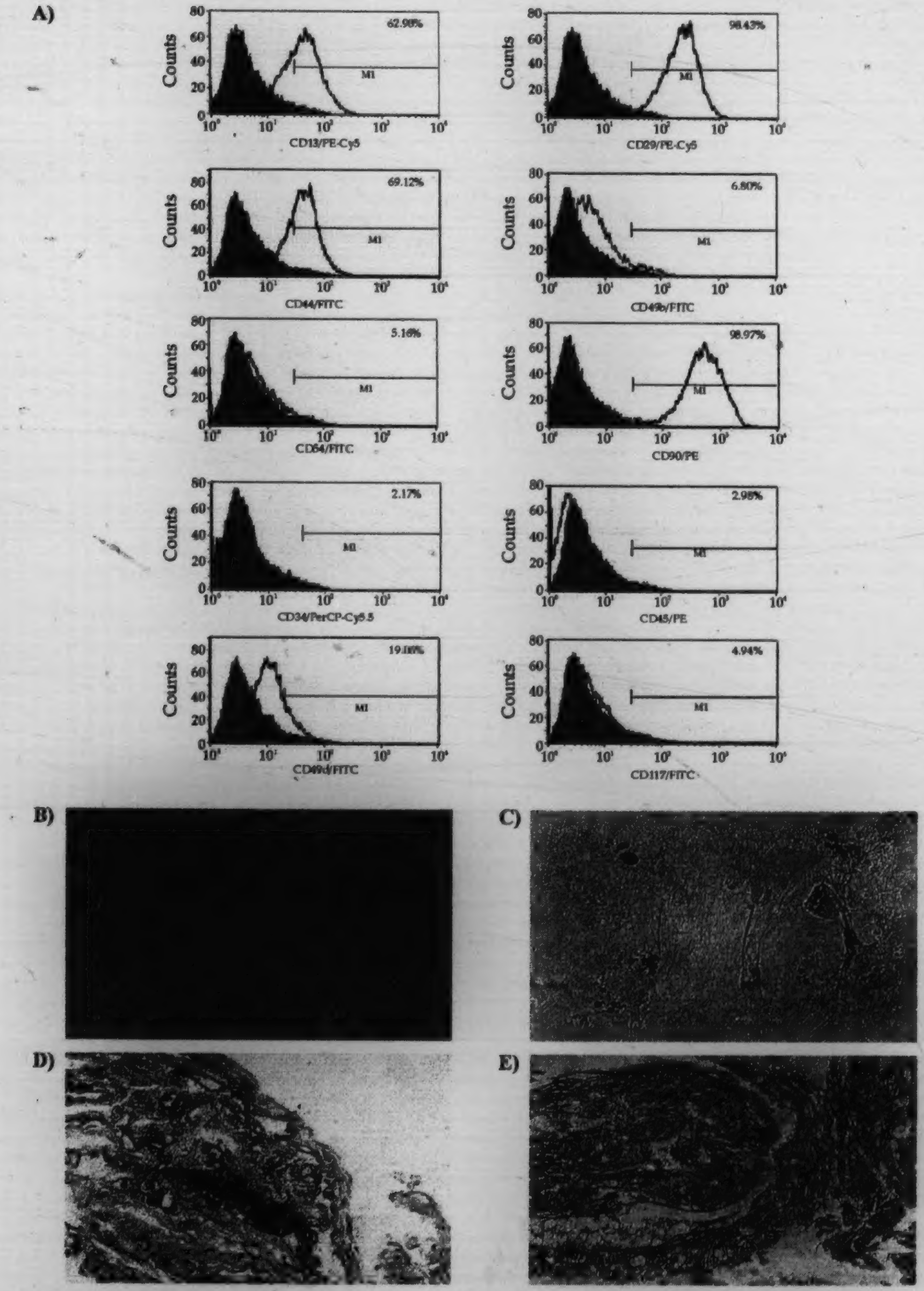

Figure 2-1: Characterization of hMSCs and demonstration of pluripotency. a) FACs analysis of hMSCs. b) hMSC, spindle-shaped morphology c) Adipogenic differentiation of hMSCs. Lipid vacuoles were visualized by Oil red O staining. d) and e) Osteogenic differentiation of hMSCs; d) Von kossa staining of mineralization (black), e) Trichrome stain for collagen (green)

Differentiation Approach #1 – Adeno-ngn3 infection

Following phenotypic and functional characterization, hMSCs were infected with adenovirus containing vectors for ngn3 and GFP, the former being a transcription factor expressed in the developing pancreas and the latter a fluorescent tag enabling visualization of infected cells, with the aim of inducing expression of transcription factors expressed during pancreatic beta cell development.

Infection efficiency was determined qualitatively by fluorescent microscopy (Figure 2-2a) and quantitatively by immunohistochemical staining of infected cells with an antibody specific for GFP. The percentage of GFP positive cells was determined by counting at least 500 cells per slide and determining the number of GFP positive cells. At time 0 uninfected cells did not express GFP, but 48hrs following adenoviral infection 56% of cells in culture expressed the fluorescent marker. GFP expression persisted for the duration of the experimental protocol, decreasing slightly with time in culture to 49% at 10 days post infection and to 33% at 20 days (Figure 2-2). We observed high variability in infection efficiency between experiments at early timepoints but with time the percentage of GFP positive cells became more consistent.

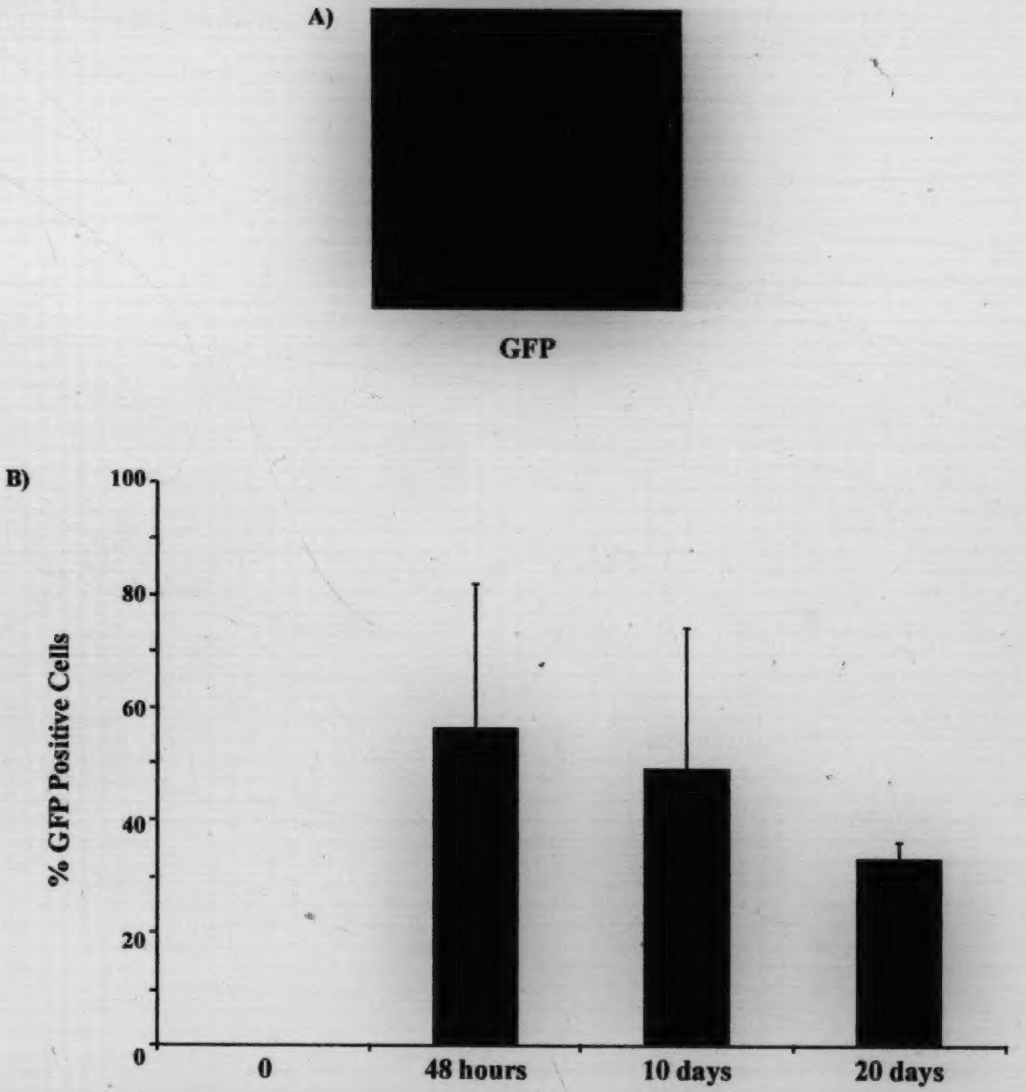

Figure 2-2: Adenovirus-mediated expression of ngn3 in hMSCs. a) Photomicrograph of GFP/ngn3 infected cells in culture. b) Long-term expression of adenoviral mediated ngn3 as shown by immunohistochemical staining for GFP tag (n=3).

Morphology

Following adenoviral infection cell morphology was altered. Prior to infection, cell cultures formed a confluent monolayer of fibroblastoid homogeneous cells. Following infection with adeno-ngn3, cells showed heterogenous morphologic changes which consisted of cells with cytoplasmic projections connecting to other cells, broad cells with striations and multiple nuclei, spheres consisting of multiple cells, and cells with dense droplets within the cytoplasm (Figure 2-3 b). Cultures infected with adeno-GFP alone did not show these morphological changes but retained the fibroblastoid morphology similar to untreated control cultures. Morphological changes and decreases in infection efficiency were noted with increasing passage number so all experiments included in this work were conducted between passages 1 and 4.

mRNA Expression

Samples from each experimental condition were taken at 48 hours, 10 days and 20 days post-infection for mRNA expression as assessed by RT-PCR. Cells infected with adenovirus containing the gene vector for mouse ngn3 showed upregulation of selective genes expressed during pancreatic endocrine cell differentiation (Figure 2-3 a). In 2 of 3 experiments mouse ngn3-dependent expression of Pax6 was observed at days 10 and 20 post-infection, whereas in the remaining experiment faint Pax6 expression was noted at 48 hours post infection. NeuroD expression was observed in all experiments, however the timing of the expression of this transcription factor varied amongst experiments, with 2 of 3 experiments showing NeuroD expression at 48 hours and 20 days post infection, but with no expression observed at 10 days post infection. The co-expression of Pax6 and NeuroD at a given timepoint indicates that there are likely cells at different stages of differentiation within these cell cultures since NeuroD is expressed downstream of Pax6 during normal pancreatic endocrine development. Two of three experiments also showed upregulation of Nkx6.1 at days 10 and 20 post-infection. Since human ngn3 was not expressed in these cultures it appears that the mouse equivalent of this gene is capable of inducing the human transcription factors that were observed in this study. We did not detect the expression of mRNA for human insulin, ngn3, Pdx1, Pax4 or Nkx2.2, nor did infection with adenovirus containing GFP alone upregulate any of the gene products noted above. These results indicate that the adenoviral vector for mouse ngn3 was inducing the upregulation of the pancreatic genes observed in these cultured hMSCs.

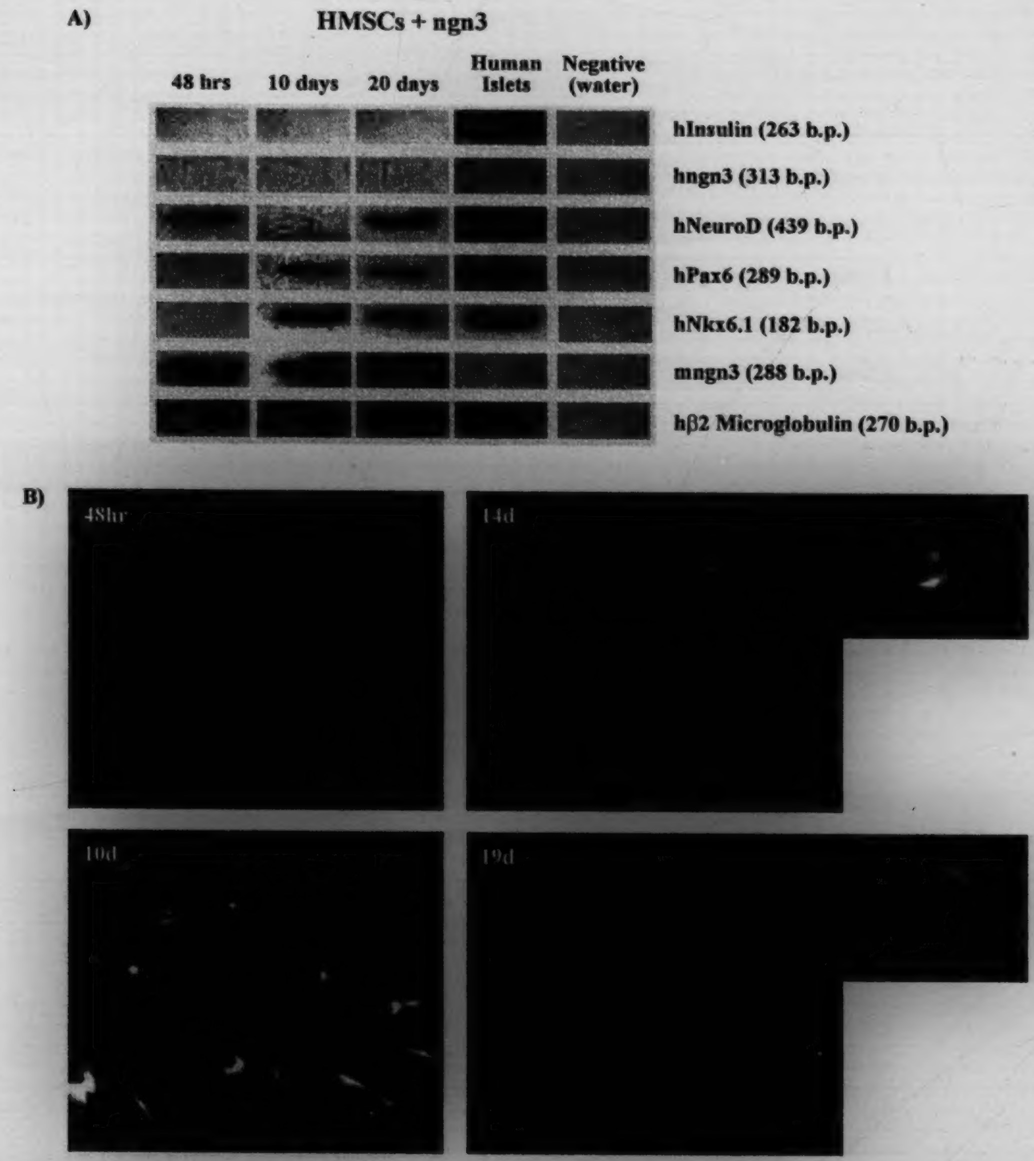
A)
HMSCs + ngn3
48 hrs
10 days
20 days
Human Islets
Negative (water)
hInsulin (263 b.p.)
hngn3 (313 b.p.)
hNeuroD (439 b.p.)
hPax6 (289 b.p.)
hNkx6.1 (182 b.p.)
mngn3 (288 b.p.)
hβ2 Microglobulin (270 b.p.)
B)
48hr
14d
10d
19d

Figure 2-3: Induced expression of pancreatic transcription factors in hMSCs and morphological changes post infection. a) RT- PCR analysis of RNA extracted from bone marrow derived hMSCs infected with adenovirus-mediated ngn3 (representative sample) compared with adult human islets. b) Morphological changes with time in culture of ngn3 infected hMSCs.

Differentiation Approach #2 – Multiple Adenoviral Infections + Media Supplementation

Based upon the morphological changes and altered gene expression observed with our first differentiation approach, we implemented a multi-step differentiation protocol consisting of Pdx1 and ngn3 gene delivery, together with growth factor media supplementation, with the aim of enhancing differentiation towards the beta cell fate.

Morphology

The first step of the supplementation protocol consisted of the addition of DMSO to culture media for the enhancement of cell permeability and infection efficiency. Immediately post infection, increased GFP expression was detected in cells cultured with the supplement protocol, indicating that DMSO did enhance infection efficiency. GFP expression was observed earlier in these cultures, however by the experimental endpoint of 30 days, infection efficiencies were not different between control and supplemented cultures.

High glucose, nicotinamide and exendin-4 were introduced into cell cultures in a timed protocol. The addition of these supplements resulted in changes in cell morphology relative to cells cultured in LG-DMEM alone (Figure 2-4b), with infected cells having round central cell bodies and narrow cytoplasmic projections compared with uninfected cells cultured in LG-DMEM which were fibroblastoid with broad flattened cytoplasm. Supplemented-uninfected cell cultures showed decreases in cellular culture confluence, indicating that supplementation decreased cell proliferation (Figure 2-4b A

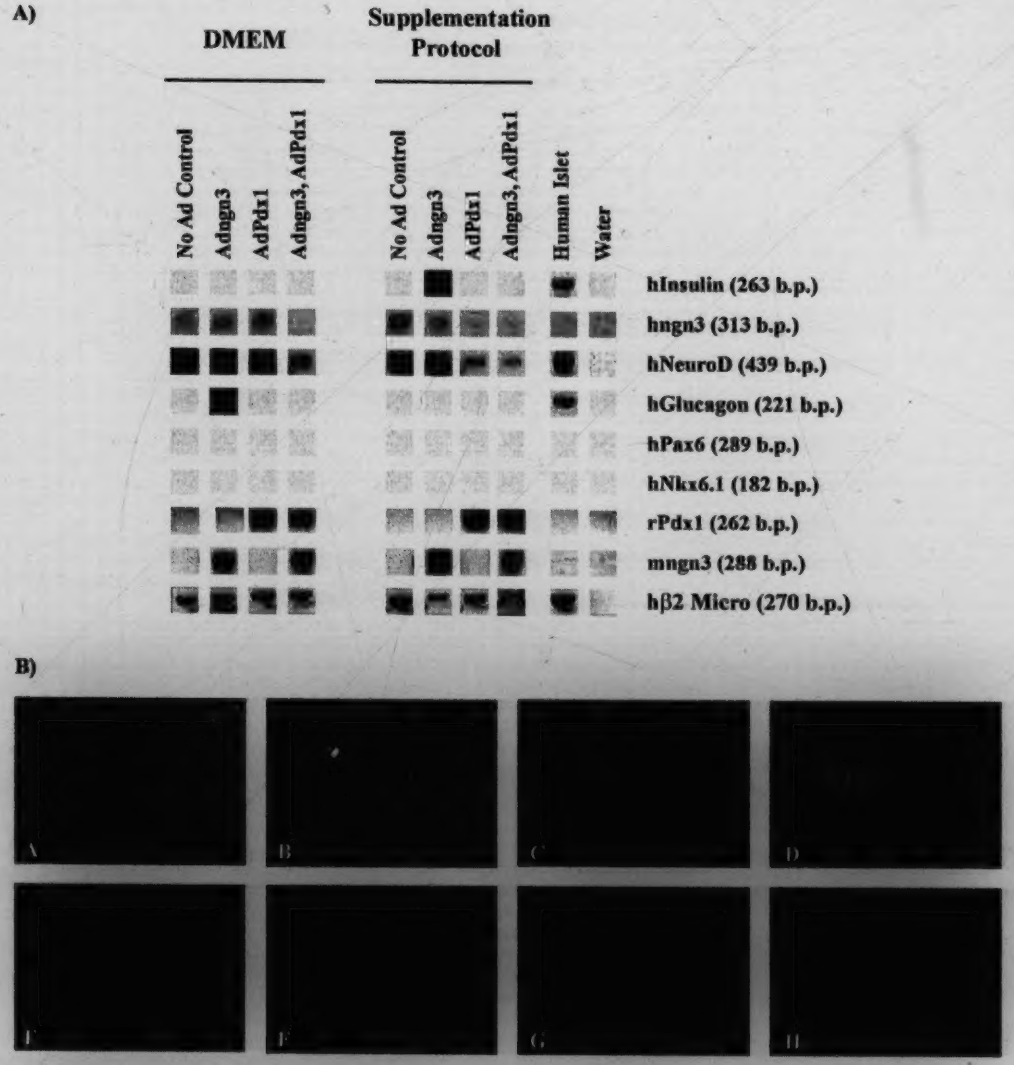
A)
DMEM
Supplementation Protocol
No Ad Control
Adngn3
AdPdx1
Adngn3, AdPdx1
No Ad Control
Adngn3
AdPdx1
Adngn3, AdPdx1
Human Islet
Water
hInsulin (263 b.p.)
hngn3 (313 b.p.)
hNeuroD (439 b.p.)
hGlucagon (221 b.p.)
hPax6 (289 b.p.)
hNkx6.1 (182 b.p.)
rPdx1 (262 b.p.)
mngn3 (288 b.p.)
hβ2 Micro (270 b.p.)
B)
A
B
C
D
E
F
G
H

Figure 2-4: Effect of adenovirus-mediated expression of ngn3 and/or Pdx1 alone and with media supplementation on key transcription factors in hMSCs. a) Representative RT-PCR analysis of mRNA extracted from bone marrow derived hMSCs treated with the indicated conditions (d27 post infection/ d30 post culture) compared with adult human islets. b) Morphological changes and cell cluster formation after cell culture and treatment with differentiation conditions; (A-D) no supplementation, (E-H) supplementation protocol; A and E – no Ad control, B and F – Adngn3, C and G – AdPdx1, D and H – Adngn3 and AdPdx1.

vs. E). With infection, cell clusters formed, with decreases in cluster size being associated with media supplementation. Also, when cells were cultured in LG-DMEM alone the cells forming the clusters were GFP positive, and with media supplementation, cell clusters contained few or no GFP positive cells.

In addition to media supplementation cells were infected with adenovirus containing Pdx1, a gene expressed by developing and mature beta cells. The addition of this gene, either alone or together with the gene for ngn3, to cultured cells yielded dramatic changes in cell morphology and orientation. By 14 days post-infection, Pdx1$^+$ or Pdx1$^+$ngn3$^+$ cells began to form rounded cell clusters, and notably, there was no heterogeneous differentiation observed when the Pdx1 gene transduction was added to the differentiation protocol - indicating that cells were induced to differentiate towards a more specific cell fate by the addition of Pdx1. Cell clusters that existed in cultures infected with Pdx1 alone, or with the combined infection of Pdx1 and ngn3, were larger than those clusters formed following ngn3 infection alone. All clusters were made up of live cells as shown by positive neural red stain (Figure 2-5b).

We found that adenoviral infection of hMSCs resulted in the expression of Pdx1 protein (Figure 2-5a). This protein was detected in cells infected with each experimental condition whereas uninfected cells did not show Pdx1 protein expression. Pdx1 protein is expressed in mature beta cells and therefore its expression in these hMSCs is a strong marker that the differentiation pathway of these cells has been altered.

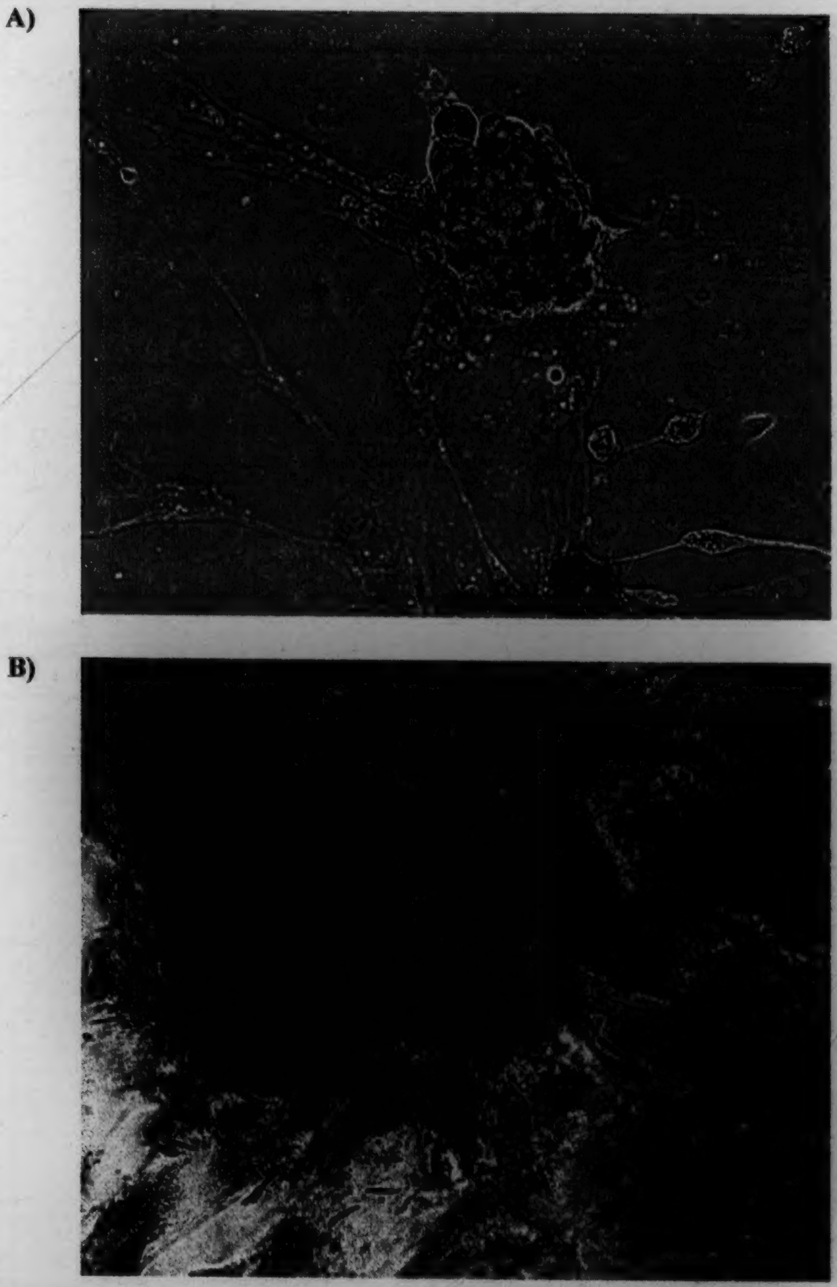

Figure 2-5: hMSC clusters stain positive for Pdx1 and neutral red. a) Cell cluster from cultures of AdPdx1 without supplementation is representative of immunohistochemical staining for Pdx1 protein. b) High magnification (320x) of cell cluster from Adngn3 without supplementation cultures shows representatively that cell clusters are composed of living viable cells as seen by their ability to take up neutral red dye from culture media.

mRNA Expression

Samples from each experimental condition were taken at 27 days post-infection and mRNA expression was assessed using RT-PCR. Cells infected with adenovirus containing the cDNA for mngn3, rPdx1, or both viral vectors showed upregulation of genes expressed during pancreatic endocrine cell differentiation (Figure 2-4). NeuroD expression was detected in all treatment groups with lighter bands detected when both viral vectors were employed. Human ngn3 gene expression was also upregulated under some experimental conditions: adeno-ngn3 with control or supplemented media, and adeno-Pdx1 transduction with control media. Of note was the detection of gene expression for glucagon and insulin in select groups of differentiated cells. With ngn3 infection alone we detected mRNA expression of glucagon, and when ngn3 infection was paired with media supplementation we detected mRNA expression of insulin - indicating that media supplementation alters the cellular differentiation from alpha-cell to beta-cell.

II-D) DISCUSSION

In this study we present evidence of three main findings. 1. Human bone marrow derived MSCs can be induced to differentiate towards the pancreatic beta cell fate. 2. Adenoviral infection with ngn3 and/or Pdx1 can stimulate MSCs to differentiate towards pancreatic islet cell fate as shown by the expression of mRNA for Pax6, Nkx 6.1, ngn3, NeuroD, glucagon and insulin. 3. Adenoviral infection with pancreatic transcription factors results in morphological changes within MSC cultures with the formation of Pdx1 positive islet-like clusters.

In this study we have presented evidence that human bone marrow derived MSCs can be induced to differentiate towards the pancreatic β cell fate. The ability of mesodermal cells, such as MSCs, to cross embryological germ layers and differentiate into pancreatic beta cells, which are of endodermal origin, has been a subject of considerable debate [3;24-27]. In this study, we have shown that when monolayers of MSCs are infected with adenoviral vectors containing gene constructs for the pancreatic transcription factors ngn3 and Pdx1 both morphological and genetic changes result. Following infection with adeno-ngn3 and adeno-Pdx1, cells within the cultures began forming cellular aggregates, with stellate aggregates forming by day 10 and spherical clusters forming by day 20 post-infection. When growth factor supplements were added to the media, cell clusters decreased in size and cells forming the clusters no longer expressed GFP. Regardless of media supplementation, cell clusters expressed Pdx1 protein, a protein that is expressed in mature beta cells and therefore a convincing marker that we successfully altered the cell fate of these MSCs.

An elegant study by Oh *et al.* (2004) also shows evidence, similar to that which we have shown here, that bone marrow derived cells can be induced to differentiate towards the beta cell fate [27]. Working with rat bone marrow, these authors report similar morphological changes to what we have shown, with cells forming spheroid clusters following 7 days of culture, and similar expression of pancreatic transcription factors and endocrine genes to what we have shown with human cells. In contrast to our work, these authors reported that the rat bone marrow derived cells could be differentiated into insulin-producing cell clusters that were responsive to a glucose challenge. We performed similar glucose challenges with our human cell clusters but found no detectible insulin secretion (data not shown). Two key differences between these studies which may account for this discrepancy in functionality are the species from which the cells were derived and the specific bone marrow cell type utilized for the differentiation work. It is possible that differences exist in the differentiation potential of MSCs across species, similar to differences that are known to exist between mouse ES and human ES cells with the former requiring leukemia inhibitory factor for maintenance of the stem cell state and the latter requiring a feeder layer to accomplish the same [28]. Secondly, a strength of our work is that we performed a thorough characterization of our cellular starting material prior to inducing cellular differentiation, thus enabling us to conclude that the MSC fraction within human bone marrow can be induced to differentiate towards the pancreatic beta cell fate. In the work discussed above with rat bone marrow, no such characterization was performed and it was the entire adherent fraction of the bone marrow which was exposed to the differentiation parameters. Experiments such as this, which start with a heterogeneous cell population, yield unanswered questions regarding the specific cell source within the bone marrow that has the potential to give rise to functional glucose sensitive insulin secreting cells.

In addition to the morphological indicators of differentiation discussed above we also detected changes in genetic expression in this study, which were indicative of MSC to beta cell differentiation. When cells were infected with ngn3 alone, genetic expression of Pax6 was observed at day 10 and 20 post infection but not at 48 hours or 27 days post infection, indicating that the cells within our cultures were undergoing a dynamic state of change with selected genes being turned on and off in a programmed way. Similar dynamic changes were seen with NeuroD and Nkx 6.1 gene expression.

We also detected concurrent expression of the transcription factors Pax6 and Nkx 6.1 which are not normally dually expressed by cells [29], indicating that cells within our cultures were at different stages of differentiation. Jensen *et al.* describe a heterogeneous population of developing pancreatic cells, showing expression profiles similar to that which we observed in the current work [29]. In studying the developing pancreas they observed some $Pax6^+ngn3^+$ double positive cells but suggest that endocrine cells derive from $ngn3^+$ cells and subsequently lose ngn3 expression. Thus Pax6 expression results from prior expression of the ngn3 gene. They also describe cells that were both $Pax6^+$ and $NeuroD^+$, which is in accordance with the mRNA profiles of the bone marrow cells in our current work.

Apelqvist *et al.* (1999) show that premature differentiation of pancreatic progenitor cells, caused by forced expression of ngn3 under the control of the Ipf1/Pdx1 promoter, results primarily in generation of glucagon-expressing cells [30]. Here we found that cells infected with ngn3 expressed glucagon mRNA when cultured in LG-DMEM, but when cells were infected with ngn3 and cultured with beta cell enhancing growth factors these cells expressed insulin mRNA in place of glucagon. Others have shown that the timing of exogenous administration of ngn3 protein is crucial for the developmental fate of the infected cells, with the differentiation pathway of the developing pancreas favoring the generation of glucagon producing alpha cells when ngn3 is administered at day e9, and the promotion of both insulin producing beta cells and glucagon producing α cells when the protein is applied at day e13.5. It is therefore clear that the timing of ngn3 administration, with respect to the developmental state of the cell, greatly influences the outcome of gene expression and differentiation of the resultant cells.

The ngn3-dependent upregulation of NeuroD reported here has also been described by others [31]. Of significance is the fact that ngn3 is not able to induce NeuroD expression in all cell types. For example previous work has shown that ngn3 cannot stimulate NeuroD expression in ARID and Panc1 cell lines. Because we see ngn3 dependent upregulation of NeuroD in bone marrow derived MSCs, we can deduce that these bone marrow cells express the cofactors (i.e. CBP/p300) and other transcription factors needed to synergize with ngn3 in order to induce NeuroD gene expression. By extension it is also therefore possible that these bone marrow cells possess the machinery necessary for full beta cell differentiation.

This work is important because it addresses and seeks to define a protocol for successful MSC differentiation into pancreatic beta cells, which if successful will provide a solution for the shortage of beta cells available for widespread islet transplantation. This study has shown positive results indicating that the conversion of MSC to pancreatic beta cells is possible. Islet-like clusters were formed from the hMSCs and beta cell gene and protein expression were detected. What is now required of future work are the key elements needed to induce functional changes in these partially differentiated, but readily accessible, cells.

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CHAPTER III

THE EFFECT OF TIMED AND SEQUENCED ADMINISTRATION OF PANCREATIC TRANSCRIPTION FACTORS ON THE DIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS

III-A) INTRODUCTION

Type 1 diabetes results from autoimmune destruction of the insulin secreting beta cells of the pancreas, with this cellular death resulting in systemic hyperglycemia. Traditional treatment for diabetic patients includes daily injection of insulin which serves to lower blood glucose levels. However, despite insulin injections, diabetes often results in late complications such as retinopathy, neuropathy, nephropathy. It has been shown that these long term complications can be alleviated by tight control of blood glucose [1], and one method for achieving regulated control is islet transplantation with the Edmonton Protocol [2]. For this procedure, islets are isolated from cadaveric donors and infused into the liver of the diabetic patient. These foreign cells function within the liver and regulate blood glycemia in a physiological way, thus protecting against the secondary complications of the disease [3].

Despite the potential therapeutic benefits of islet transplantation, its widespread applicability is limited by the shortage of donor islets. If this treatment option is to become a widespread form of therapy for type I diabetic patients the supply of human organs will be a major limitation, thus alternative tissue sources are being explored.

Adult stem cells hold the potential to alleviate this problem of tissue supply as they may have the potential to differentiate into insulin secreting cells, which could be used for islet transplantation [4-9]. Adult stem cells generally lack tissue-specific characteristics, but can be induced to differentiate into specialized cells with appropriate stimuli [10], such as cell turnover, wound signaling, or disease. Bone marrow derived mesenchymal stem cells (MSCs) are adult stem cells that hold much promise for therapeutic use as they can be easily isolated from live donors and the cells show considerable capacity for expansion and differentiation across primary germ layers. Many reports describe the differentiation of MSCs into bone, cartilage, fat [10], skeletal muscle [11], cardiomyocytes [12], neuronal cells [13-15], and hepatocytes [16]. These cells have also been shown to have the ability to differentiate into insulin secreting cells [4;7]. However complete beta cell differentiation has yet to be achieved.

Published methods for mesenchymal stem cell differentiation into insulin secreting cells center largely on the in vitro administration of growth factors to induce differentiation [17-20], or on the in vivo transplantation of the cells with or without organ damage [21;22]. To date however, a reliable and reproducible method for differentiating human adult stem cells into glucose responsive insulin secreting cells has not been established. These shortfalls may be due to deviations, in previous protocols, from the sequence of genetic events which occurs during normal pancreatic embryogenesis.

Adenoviral transduction with pancreatic genes has been shown to be an effective approach for directing stem cell differentiation towards the pancreatic fate in other cell systems. In this differentiation method, embryological events are mimicked and developmental genes are administered in an attempt to guide stem cell differentiation. Working with human pancreatic ductal cells Heremans *et al.* demonstrated that adenoviral infection with engineered vectors containing ngn3, a transcription factor expressed during pancreatic development, resulted in insulin gene expression [23]. Genetic delivery for the directed differentiation of stem cells, administers key factors that are expressed during pancreatic development as a means of guiding cell differentiation pathways. It is therefore possible that such an approach, combined with physiological differentiation signals such as organ damage, may provide differentiation signals sufficient to induce complete beta cell differentiation.

In this study we have devised an innovative differentiation protocol that involves the sequential and timed administration of multiple adenoviral vectors bearing genes (Pdx1, ngn3, NeuroD, and Pax6) expressed during pancreagenesis as a means to induce bone marrow-derived MSCs to differentiate towards the pancreatic beta-cell fate. Our differentiation approach is therefore to mimic as much as possible the events which occur during embryogenesis in an attempt to differentiate adult MSCs into insulin secreting cells.

III-B) METHODS

All experimental protocols used in this study were approved by the Research Ethics Board of the University of Alberta.

Cell Culture

Bone marrow- derived MSCs were obtained from Cambrex Bioscience Walkersville Inc (Walkersville, MD, USA). Human MSCs, used for the differentiation protocols, were obtained from the normal bone marrow of a 32-year-old Caucasian and a 19-year-old Asian male. MSCs were plated at a density of 5000 cells/cm^2 and cultured with Mesencult growth medium with Stimulatory Supplements (Stem Cell Technologies, Vancouver, BC, Canada) at a volume of 0.5 mL media/cm^2 and experiments were performed between passages 1 and 5. Media was replaced every 2-3 days and cells were passaged at 70-80% confluence. Prior to the implementation of experimental differentiation protocols, MSCs were characterized for cell surface markers and differentiation potential (adipogenesis and osteogenesis) to ensure the function and phenotype of the starting material (details of the characterization procedure and results are described in Chapter II).

Adenoviral Transduction and Cell Differentiation

Following a thorough characterization of the MSCs, experimental differentiation strategies were employed with the aim of differentiating MSCs toward the pancreatic beta cell fate, by mimicking the sequence of transcription factor activation known to occur during pancreatic embryogenesis. The timing, sequence and combination of viral administration were selected based upon genes that are known to be expressed in the developing pancreas, with Pdx1 and Pax6 expressed early in development followed by expression of ngn3, NeuroD and re-expression of Pdx1. To achieve this, human bone marrow derived MSCs were transduced, using a modification of a previously described protocol [24], with multiple replication-deficient adenoviruses containing a vector with the genes for enhanced green fluorescent protein (eGFP) and one of Pdx1, ngn3, NeuroD or Pax6, driven by separate CMV promoters (gifts from Dr. Harry Heimberg, Free University of Brussels, Belgium). For the infection, adherent MSCs (70-80% confluence) were trypsinized using 0.25% trypsin-EDTA and cell number was determined using a hemocytometer. Cells were plated in tissue culture treated plates and infected at a multiplicity of infection (MOI) of 10 with each adenovirus for 4 hours at 37°C in 86 $\mu L/cm^2$ HBSS. Cells were subsequently washed two times with HBSS to remove virus particles and re-cultured with LG-DMEM media containing 100 U/mL penicillin and 100 μg/mL streptomycin.

Five sequential adenoviral infections were performed resulting in a cumulative MOI of 50. In vitro studies included two separate infection sequences based upon published data indicating timing discrepancies in the expression of Pax6 during pancreogenesis, with some reports presenting evidence of early Pax6 expression and others reporting late Pax6 genetic expression. Infection sequence #1 was: day 0 - Pdx1 and Pax6, day 5 – ngn3, day 6 - NeuroD, and day 7 – Pdx1. Infection Sequence #2 was: day 0 – Pdx1, day 5 ngn3, day 6 - NeuroD and Pax6, and day 7 – Pdx1. Subsequent in vivo studies, involved transductions sequenced as in infection sequence #1 for all experiments.

In Vitro Studies

For *in vitro* studies, cells were plated into 12-well tissue culture treated plates at a density of 24221 cells/cm^2, and the infection sequence (detailed above) was initiated immediately for experimental cells, while control cells were cultured without infection. Control and infected cells were also cultured with and without 3nmol Activin A (R&D) in LG-DMEM (Gibco) media with 100 U/mL penicillin and 100 μg/mL streptomycin to determine the effect of this growth factor on beta cell differentiation. Control and experimental cell preparations were cultured for up to 20 days with cultures assessed for morphology, gene expression by RT-PCR and protein expression by immunohistochemistry at days 10 (n = 5) and 20 (n = 3) post-initiation.

In Vivo Transplantation Studies

Prior to transplantation, cells were plated at a density of 24 x 10^3 cells/cm^2 in 150mm tissue culture treated plates with LG-DMEM media and were cultured as uninfected controls or as adenoviral infected treated cells (infection procedures detailed above). Following 12 days of culture, adherent cells were removed from culture plates using a cell scraper and the graft was characterized for DNA, insulin, mRNA, and protein expression by immunohistochemistry. To determine cell number, aliquots of the cell suspension were measured in duplicate for total cellular DNA content using a Hoefer DyNa Quant 200 fluorometric assay (Amersham Pharmacia Biotech, San Francisco, CA, USA) as described previously [25]. Insulin content was measured after cellular extraction by ELISA [25].

Prior to transplantation the cells were aspirated into polyethylene tubing (PE-50), and pelleted by centrifugation. Infected and control MSCs were then transplanted into the pancreatic remnant of Halothane-anesthetized 6-8 wk old C57BL/6-rag1$^{-/-}$ knockout mice (RagB6; purchased from Jackson Laboratories, Bar Harbor, Maine, USA) that had undergone partial pancreatectomy and full splenectomy immediately prior to graft infusion. The pancreas was reached via a vertical midline incision and approximately 50% of the organ was removed following organ ligature at the midpoint. The graft was gently injected into the pancreas using a micromanipulator syringe and PE-50 tubing. Once the tubing was removed, the injection site was cauterized with a disposable high-temperature pen (Aaron Medical Industries, St. Petersburg, FL). Graft size (mm of tissue pelleted within the PE tubing) and mouse weight were measured using a standard ruler and balance. The excised portion of the pancreas was snap frozen in liquid nitrogen and the insulin content was determined after organ extraction by RIA and compared to that of a whole mouse pancreas [25]. A third group of animals underwent surgical pancreatectomy, but did not receive a cellular graft infused in the pancreatic remnant.

Nonfasting blood glucose was measured on the day of surgery, one day post surgery, and weekly thereafter between 9:00 and 11:00AM. Blood samples were withdrawn from the tail vein and glucose levels were determined with a portable glucose meter (One Touch; Lifescan Inc., Milpitas, Canada). At days 7 (n = 4 infected, 2 control, 1 pancreatectomy), 20 (n = 4 infected, 2 control, 1 pancreatectomy) and 40 (n = 2 infected, 2 control, 1 pancreatectomy) post-transplant, animals from each experimental group underwent pancreatectomy to remove the MSC graft, or pancreatic remnant. Prior to graft removal, animals were fasted for 3 hours followed by the administration of a dextrose solution (3mg/g body weight) by oral gavage. Glycemia was measured at 0 and 15 minutes followed by cardiac puncture, and serum collection for human c-peptide detection using an Ultrasensitive c-Peptide ELISA kit (Mercodia AB, Uppsala, Sweden). Grafts were located in the mouse pancreas by examining the area of the transplant for the cauterization scar, and subsequently locating the human tissue, which was yellow in colour as compared to the surrounding mouse tissue. Grafts were trimmed and either placed in Z-fix for > 24 hours followed by paraffin embedding, sectioning into 3 μm thick sections and placed onto histobond slides, or suspended in Trizol reagent as described in the "RT-PCR analysis" section for subsequent molecular analysis.

Cell Characterization

Phase Contrast and Fluorescent Microscopy

Cell morphology and eGFP expression were characterized throughout the course of the differentiation period by phase contrast microscopy using a Zeiss Axiovert 25 inverted fluorescent microscope (Carl Zeiss Canada, ON, Canada) with a Coolsnap camera and IP lab software (Scanalytics Inc., Fairfax, USA).

RT-PCR

Samples for mRNA analysis were pelleted, suspended in Trizol reagent (Invitrogen, Carlsbad, CA, USA) and stored at -80°C for molecular analysis. Samples from transplanted grafts were broken up, in Eppendorf tubes, using microcentrifuge pestles (Rose Scientific, Edmonton, Canada) before the addition of Trizol and subsequent freezing. For all mRNA processing, samples were thawed and RNA extracted according to the manufacturer's protocol (Gibco). cDNA was constructed from 1 μg mRNA with 10 units (200 U/μl) TaqMan reverse transcriptase (Applied Biosystems, Branchburg, New Jersey, USA) in 1X buffer containing 0.01M DTT, 0.5 mM dNTPs, and 0.02 μg/μl oligo dT15. For each sample, 2 μl of cDNA was used per 30 μl reaction along with 1X PCR buffer, 2 mM MgCl, 0.2 mM dNTPs, 1 unit Platinum taq polymerase (5 U/μl), and 0.5 μM of each primer pair. Primer pairs employed included: insulin, ngn3, Pdx1, Synaptophysin, Glucagon, Nkx2.2, Nkx6.1, Pax4, Pax6, NeuroD, and β2 microglobulin. All chemicals/reagents were from Gibco/Invitrogen and primer sequences are detailed elsewhere [23;26;27]. For PCR reactions, 35 cycles were performed, with 30 sec. denaturation at 94°C, 30 sec. annealing at 58°C, and 30 sec. extension at 72°C. Final polymerization was at 72°C for 10 minutes followed by a 4°C hold. Products were separated on a 2% ethidium bromide stained agarose gel and images captured with Alpha Digidoc software (Perkin-Elmer, Boston, USA). β-2 microglobulin primers were used to verify the absence of genomic DNA contamination in samples as only the 268 b.p. fragment was seen and not the 900 b.p. genomic version of the gene. Samples of human islets were used as positive controls for genes examined, and negative controls consisted of Sigma water (Sigma, Oakville, ON, Canada) in place of experimental cDNA.

Immunohistochemistry

In vitro studied cells were sampled for immunohistochemical analysis at experimental timepoints (day 10 and day 20) and prior to transplantation. For morphological assessment, intact cellular aggregates were washed with phosphate buffered saline (PBS) and stored in 1mL Z-fix (Anatech Ltd, Battle Creek, MI) for at least 24 hours prior to agar embedding. Samples were subsequently embedded in 200 μL of a 2% low melting point agarose solution and allowed to harden in tissue pellet molds at 4 °C before processing, paraffin embedding, and sectioning (3 μm) onto histobond slides. Harvested, *in vivo* MSC grafts were stored in z-fix for at least 24 hours before processing, paraffin embedding and sectioning as described above.

Immunostaining of *in vitro* and *in vivo* samples was performed using the ABC-DAB method (Avidin-Biotin complex visualized with the chromagen diaminobenzidine). Sections were quenched with 20% H_2O_2/80% methanol solution to eliminate non-specific staining. Antigen retrieval for human mitochondria staining involved microwaving for 12 minutes at 80% power (Sanyo household model, 1260W) in 800 mL Na^+ Citrate. Blocking was performed with 20% normal goat serum (Fisher) for 15 minutes. Primary antibody concentration for mouse anti-human mitochondria was 1/100 and incubation was for 30 minutes at room temperature followed by wash (3X) with PBS before addition of the secondary antibody. The biotinylated secondary antibody was obtained from Vector Laboratories (Burlingame, USA) and used at a concentration of 1/200 for 20 minutes. ABC complex (Vector) incubation time was 40 minutes and visualization was with the chromagen diaminobenzadine (Biogenex, San Ramon, USA) for 5 minutes. All images were captured on a Zeiss Axioskop II fluorescent microscope (Carl Zeiss Canada) with a Coolsnap camera and IP lab software (Scanalytics Inc.).

Data Analysis

All values are reported as mean ± SEM of n independent observations. Statistical significance was calculated with a two-tailed unpaired Student's t-test or a one-way ANOVA and Scheffe's test in the case of multiple comparisons, using SPSS 14 software

(SPSS Incs., Chicago, Illinois). Significance levels were set at $P<0.05$ unless indicated otherwise.

III-C) RESULTS

Adenoviral transduction and *in vitro* assessment of hMSCs

Morphology

Adenoviral transduction of hMSCs, with key genes expressed during pancreatic embryogenesis, was performed to determine the impact of genetic delivery on the differentiation of hMSCs into insulin secreting beta cells. Cells were infected over the course of 7 days, with viral vectors that were administered using a timed and sequenced protocol designed to mimic that which occurs during embryogenesis. Following the infection sequence changes in cell morphology resulted; at day 5 of the protocol, small cell clusters began to form in experimental cultures while control cultures retained fibroblastoid morphology. By day 10, experimental cultures consisted of stellate cell clusters (Figure 3-1 A), which continued to aggregate from day 10 to day 20, at which time cultures consisted of spherical islet-like cellular aggregates composed of eGFP positive and negative cells (Figure 3-1 E). These cell clusters resembled human islets (Figure 3-1F) and islet-like clusters generated *in vitro* from pancreatic stem cells [28]. Uninfected control cultures retained fibroblastoid morphology throughout the entire culture time (Figure 3-1 B,D).

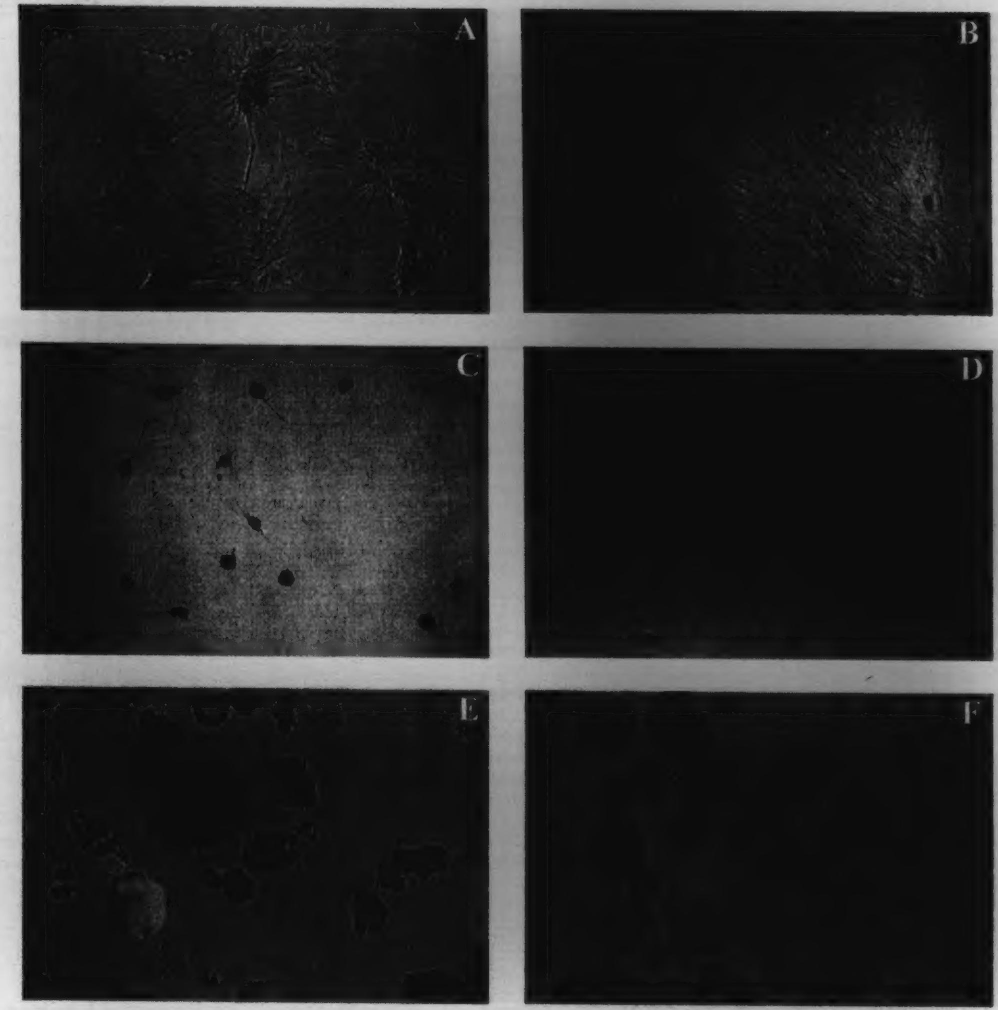

Figure 3-1: Morphology of transduced (A, C, E) and control (B, D) human MSCs at 10 days (A,B) and 20 days (C,D) following the initiation of the infection sequence. (E) MSC-derived islet-like clusters in suspension. (F) human islets.

Two variables, the addition of activin A and the timing of Pax6 within the viral infection sequence, were assayed to determine the impact of these factors on hMSC differentiation. Infected cells cultured with activin A, showed GFP expression slightly earlier than those cells cultured without this supplement. However by the experimental end point of 20 days both culture conditions showed similar GFP expression. Activin A did induce earlier cell cluster formation in infected cultures, and the addition of this supplement resulted in larger cluster size by day 20 (Figure 3-2). The timing of Pax6 gene transduction, at day 0 or day 6 of the protocol, did not impact the cellular morphology of the infected cultures.

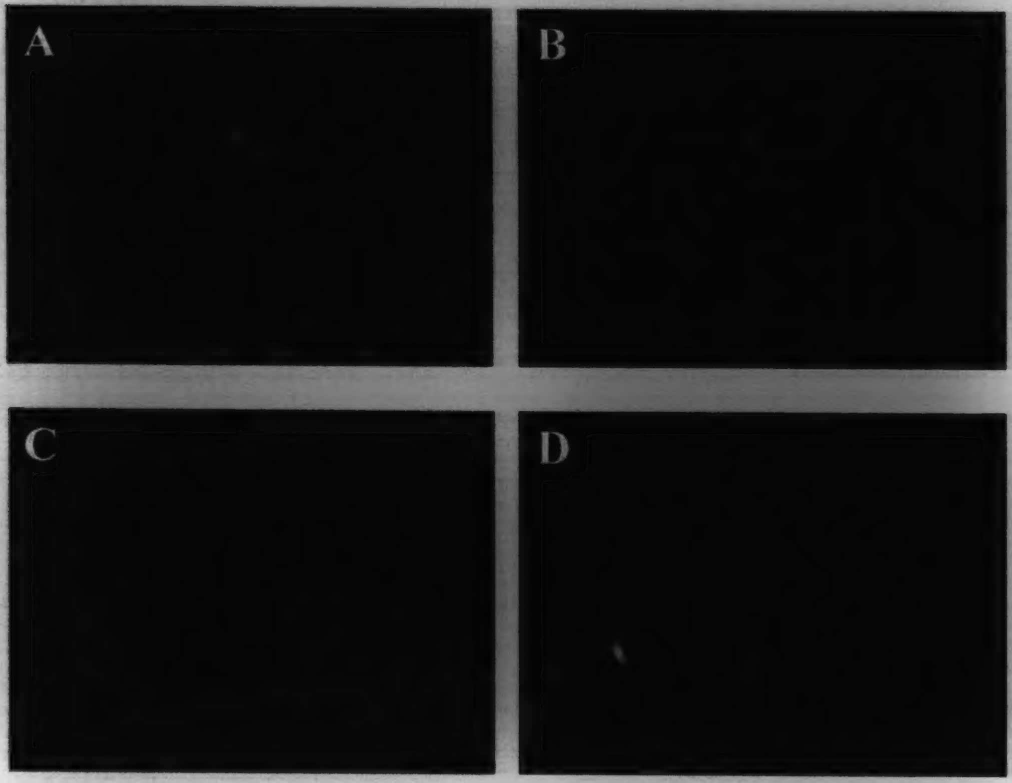

Figure 3-2: Cell cluster formation in transduced human MSCs cultured without (A,B) and with activin A media supplementation (C,D).

mRNA Expression

Samples from each experimental condition were taken at days 10 and 20 following the start of the infection protocol and mRNA expression was assessed using RT-PCR. Cells, infected with viral sequence #1 and cultured without the addition of activin A to culture media, showed upregulation of a number of genes which are expressed during pancreatic endocrine differentiation (Figure 3-3). Differentiated cells showed mRNA expression of ngn3, glucagon and synaptophysin at day 10 and expression of glucagon, synaptophysin, Pax6, and Nkx6.1 at day 20. Strong expression of NeuroD was noted in all experimental conditions. Gene expression of human insulin, Pdx1, Nkx2.2, and Pax4 were not detected. The addition of activin A or the transduction with viral sequence #2 did not yield these numerous changes in gene expression. Because viral sequence #1 yielded enhanced pancreatic gene expression compared with other assayed parameters, this protocol was adopted for subsequent *in vivo* studies.

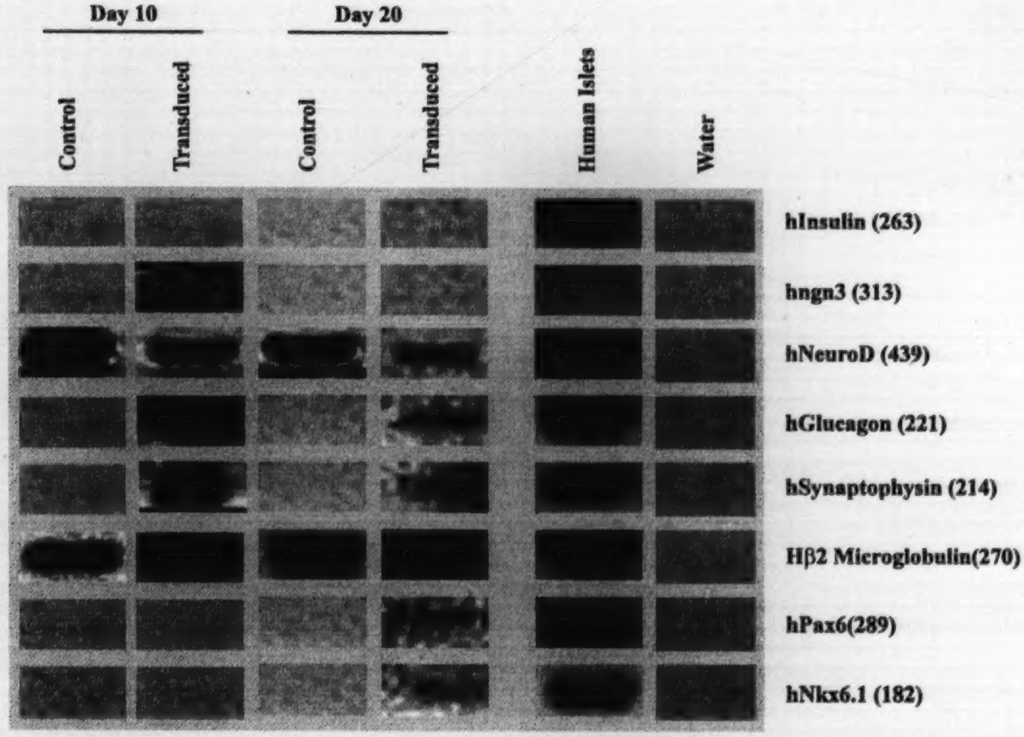
Day 10
Day 20
Control
Transduced
Control
Transduced
Human Islets
Water
hInsulin (263)
hngn3 (313)
hNeuroD (439)
hGlucagon (221)
hSynaptophysin (214)
Hβ2 Microglobulin(270)
hPax6(289)
hNkx6.1 (182)

Figure 3-3: RT-PCR analysis of human MSC samples from transduced and control *in vitro* cultures. Samples represent gene expression from samples at day 10 (n = 4 control, n = 4 transduced) and at day 20 (n = 2 control, n = 2 transduced) cultures.

In Vivo Transplantation Studies

Based upon the observed morphological and genetic changes of *in vitro* adenoviral infected hMSCs, we sought to complete the pancreatic differentiation cascade by transplanting these hMSCs into the pancreatic remnant of partially pancreatectomized RagB6 mice. Control and infected hMSCs were analyzed for DNA and insulin content prior to transplantation, with an average of 0.2×10^6 infected cells and 0.6×10^5 control cells transplanted per animal, and all grafts containing no insulin prior to transplantation as measured by RIA.

Partial-pancreatectomy was performed prior to MSC graft infusion, with the pancreatic remnant snap frozen followed by the quantification of insulin content of the remnant by organ extraction and RIA. The insulin content of a whole mouse pancreas taken from a RagB6 mouse was 27.29 µg, and the average insulin content of the pancreas remnants removed prior to transplantation was 9.72 µg, indicating that on average 35.62 % of the endocrine pancreas was removed prior to graft infusion. Following pancreatectomy, all animals exhibited elevated blood glucose at day 0 in the range of 13.86 mmol/L (infected) to 16.77 mmol/L (pancreatectomy only). At days 1 and 14 post transplantation, animals that received experimental MSC grafts, showed blood glycemia that was significantly reduced compared to control animals (Figure 3-4). Mice that received experimental infected MSC grafts also showed tighter blood glucose regulation throughout the 41 day post-transplant period compared with animals receiving control MSCs.

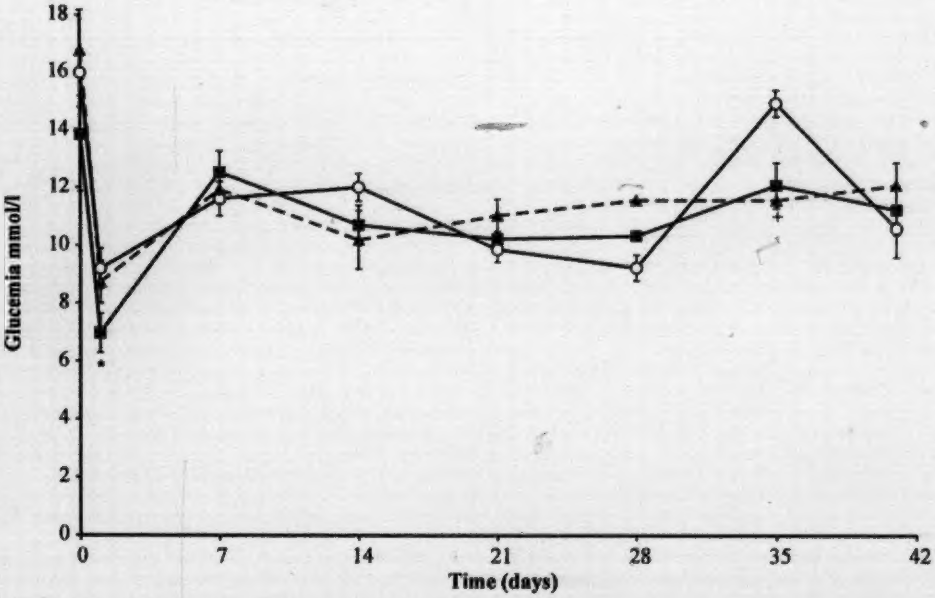

Figure 3-4: The effect of human MSC transplantation on daily blood glucose values of RagB6-/- mice. Blood glucose levels (mmol/L) in 16 mice transplanted with either infected MSCs (■ n = 10) or non-infected control MSCs (o n = 6), or who had underwent pancreatectomy alone (▲ n =6). At least 2 animals from each condition underwent pancreatectomy for graft removal at 7, 21 and 41 days post-transplant. * (α .05), + (α .10)

Prior to graft removal, mice were given an oral bolus of dextrose. The elevation in blood glucose 15 minutes following dextrose administration did not differ between those animals transplanted with control or infected hMSCs. Hyperglycemia of control mice was 14.8 ± 2.45 mmol/L, while that of experimental mice was 13.78 ± 2.64 mmol/L. Similarly, mice from each experimental group did not differ in body weight throughout the post-transplant follow up, with those animals receiving control hMSCs showing a weight gain of 2.48 ± 0.43g and those receiving infected hMSC grafts gaining an average of 2.56 ± 1.74g. Immediately prior to graft harvest, whole blood was collected and the serum assessed for human c-peptide. Low levels of c-peptide were detected in 2 of 4 experimental mice at 7 days post-transplant and in 100% of experimental mice at day 20 post-transplant. No peptide was detected at day 40 in experimental mice. No c-peptide was detected at any experimental timepoint in animals receiving control hMSCs or in mice who were partially pancreatectomized but did not receive a human cell graft (Table 3-1).

Table 3-1. Effect of hMSC Transplantation on the Concentration of Human Serum C-Peptide in RagB6 Mice

Condition	Concentration of human serum c-peptide (pmol/L)		
	Day 7	Day 20	Day 40
Transduced hMSCs	0.00, 0.00, 0.45, 1.86	0.52, 0.59, 1.01, 1.23	0.00, 0.00
Control hMSCs	0.00, 0.00	0.00, 0.00	0.00, 0.00
Pancreatectomy Alone	0.00	0.00	0.00

Values represent data from individual subjects.

Grafts were recovered from control and experimental animals at 7, 20 and 40 days post-transplantation, with graft detection facilitated by the difference in colour between human and mouse tissue - with human tissue being more yellow than mouse (Figure 3-5). Immunohistochemical analysis of recovered grafts, for the detection of human mitochondria, confirms that the human cell grafts persist within the mouse pancreas throughout the post transplant period (Figure 3-6). Graft mRNA expression was assessed using RT-PCR, and grafts from both control and experimental animals showed expression of human synaptophysin (Figure 3-7). This gene expression was also noted *in vitro*, however expression was faint and was restricted to transduced cells. Transplanting hMSCs into the regenerative environment of a partially excised pancreas therefore likely provides cellular signals necessary for synaptophysin expression in both transduced and control cells. NeuroD was expressed in experimental grafts recovered at 7 and 20 days post transplantation, but was not detected in experimental grafts at 40 days post-transplant. In vitro, the expression of NeuroD was noted in both control and experimental cells; it therefore appears that the regenerating pancreas is inhibiting NeuroD gene expression in control cells post-transplantation, while adenoviral transduction results in persistent NeuroD expression. Human insulin, ngn3, Pdx1, glucagon, Nkx2.2, Nkx6.1, Pax4, and Pax6 gene expression were not detected in MSC grafts.

Figure 3-5: Transduced intra-pancreatic human MSC graft removed at 7 days post-transplant. Human graft (arrow) is identified by a yellowish colour as opposed to lighter colored murine pancreatic tissue.

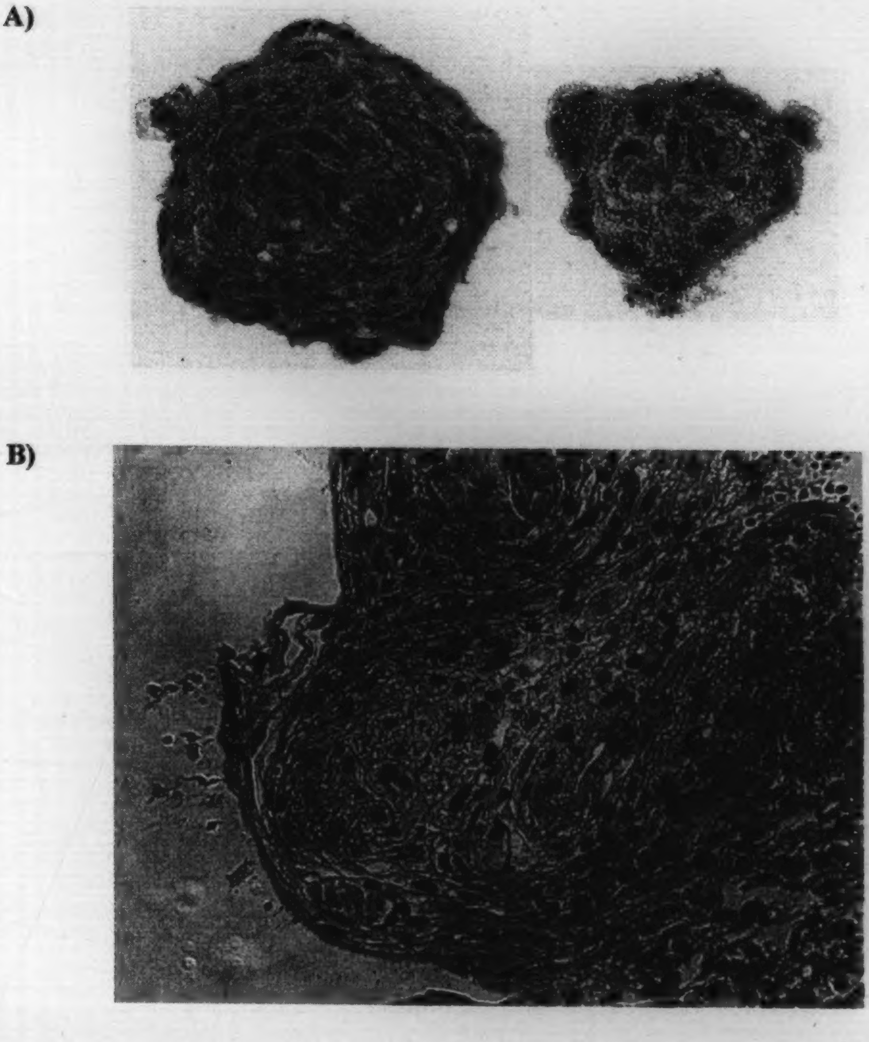

Figure 3-6: Immunostaining of human MSCs for the expression of human mitochondria (brown), pre-transplantation (A) and in grafts removed at day 7 post-transplant (B).

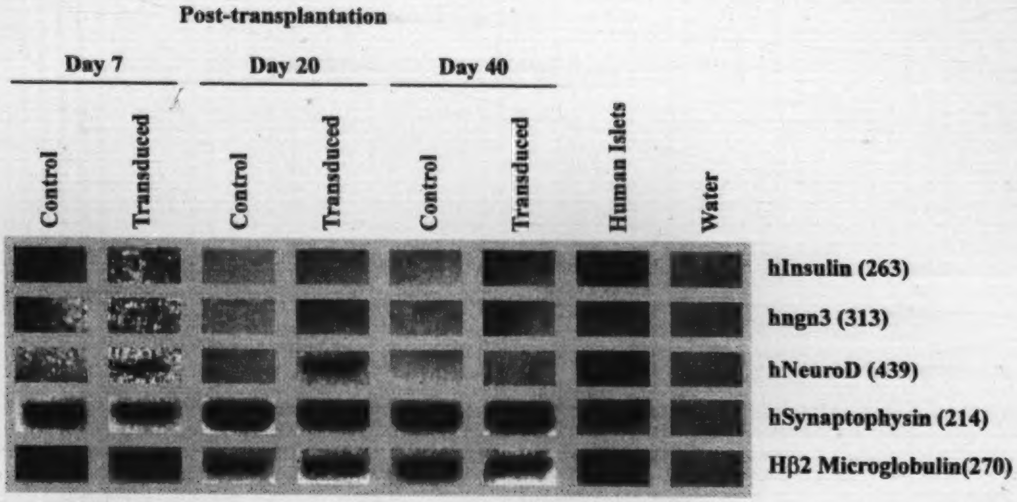
Post-transplantation
Day 7
Day 20
Day 40
Control
Transduced
Control
Transduced
Control
Transduced
Human Islets
Water
hInsulin (263)
hngn3 (313)
hNeuroD (439)
hSynaptophysin (214)
Hβ2 Microglobulin(270)

Figure 3-7: RT-PCR analysis of human specific gene expression in transduced and control human MSCs transplanted into the pancreatic remnant of mice to which partial pancreatectomy was performed immediately prior to graft transplantation. RNA/cDNA integrity was confirmed by detection of the housekeeping gene β-2-microglobulin.

III-D) DISCUSSION

In this study we present evidence of three main findings. 1. Adenoviral infection of hMSCs with pancreatic genes, timed and sequenced to mimic that which occurs during pancreatic embryogenesis, results in the formation of islet-like cell clusters. 2. hMSCs, differentiated using this protocol, express pancreatic genes both *in vitro* and *in vivo*. 3. Following hMSC transplantation into the regenerating pancreas, human c-peptide was detected in the serum of mice receiving grafts of hMSCs transduced with pancreatic genes.

In this study we show that hMSCs can be induced to differentiate towards the pancreatic beta cell fate, following directed differentiation using adenoviral transduction with pancreatic genes and transplantation of cells into the regenerating pancreas. There have been reports that rodent MSCs can be induced to express beta cell mRNA [4;9;29] and to secrete insulin in a glucose sensitive way [4]. However these cells still produce less insulin than normal pancreatic islet cells and the reproducibility of these protocols has been questioned [30;31]. Work with human MSCs has yielded results which, while being more consistent, have not reported full beta cell differentiation or insulin secretion from the differentiated cells [7;32]. The current study has examined the capacity of hMSCs to differentiate into insulin secreting beta cells both *in vitro* and *in vivo* and is unique in that the potential of these human cells to regulate glycemia *in vivo* has been examined. We chose to direct hMSC differentiation at the genetic level by administering genes for Pdx1, Pax6, ngn3, and NeuroD which are known to be key genetic determinants during pancreatic embryogenesis.

Following viral transduction, hMSCs formed stellate cell aggregates which became spherical islet-like clusters by the end of the differentiation period. These morphological changes are of significance because while there have been reports of islet-like clusters generated from rodent MSCs [4;9;29], such morphological changes have not been observed until now in human MSCs [7;32], although neoislet generation has been reported from monocyte human cell cultures [33]. In our work, stellate islet-like clusters were observed following adenoviral infection after 10 days of culture and spherical islet-like clusters after 20 days, which are significantly shorter time spans than have been reported for rodent cells following media supplementation to induce differentiation [9], where stellate clusters formed following one month of culture and spherical clusters appeared after 2-4 months of culture. From these differences it appears that adenoviral transduction yields expeditious differentiation, relative to media supplementation, as an initiator of beta cell differentiation in mesenchymal stem cell cultures.

Morphological changes, similar to those found here, were reported by Ramiya *et al.* (2000) who found that expanded pancreatic ductal epithelial cells could be differentiated into islet-like clusters [28]. Since this report, our group has defined that a mesenchymal stem cell population exists within the adult ductal epithelial cells of the pancreas [27]. It is therefore possible that the earlier reports of Ramiya were in fact demonstrating mesenchymal stem cell to islet differentiation in pancreatic mesenchymal stem cells.

The islet-like clusters observed here show genetic changes which indicate that differentiation towards the pancreatic beta cell fate has been initiated. Transduced cells show faint gene expression of ngn3, glucagon, synaptophysin, Pax6 and Nkx6.1. Partial genetic expression in differentiated rodent and human MSCs is common in studies that have sought to differentiate these cells towards the beta cell fate. Rat and mouse MSCs have been shown to express the pancreatic genes for insulin and GLUT2 [4;9] while simultaneously lacking gene expression of Pdx1 [4] and NeuroD [9]. Human adipose derived MSCs, induced to differentiate using a combination of soluble factors, also expressed ngn3 and glucagon post differentiation [32], similar to the gene expression noted in the present study. Currently there is no genetic consensus, between various works, regarding which genes show more evidence of success when aiming to induce MSC to beta cell differentiation. However, the genetic profile of our study is very similar to that reported in human monocyte derived neoislet clusters [33] where both studies show genetic expression of ngn3, Nkx6.1, NeuroD and Pax6 in differentiated islet-like cells.

Following transduction and hMSC transplantation into the regenerating pancreas, we observed human c-peptide within the serum of recipient mice. C-peptide is a cleavage product, generated during insulin protein synthesis, and is a reliable surrogate marker for insulin production. Evidence, presented here, of this peptide within mouse serum shows convincingly that the transplanted hMSCs had generated human insulin. Ruhnke *et al.* (2005) reported c-peptide expression in neoislets derived from human monocyte cultures [33]. Neoislets were transplanted beneath the kidney capsule of diabetic mice and hyperglycemia was reversed within 2 days of transplantation. In the current study we did not observe overall reversal of hyperglycemia following experimental hMSC transplantation, however glycemia was reduced in this treatment group compared to controls immediately post-transplantation and at 35 days post-transplant. It is possible that transplanted cells within our study differentiated to a degree sufficient to synthesize c-peptide, but that cells did not differentiate to a point where they possessed the cellular machinery necessary for the secretion of insulin and the c-peptide product into circulation. Since we did not see large scale reversal of hyperglycemia it is possible that c-peptide was released from cells following cell death, which may have resulted from insufficient blood vessel formation and nutrient delivery to the graft. Some differences between our work and that involving functional monocyte-derived neoislets, which may explain the discrepancies between results, include that our hMSCs cells were transplanted into the regenerating pancreas while the monocyte derived neoislets were transplanted beneath the kidney capsule. We chose to transplant cells directly into the regenerating pancreas because previous work has shown that following pancreatectomy, islets within the mouse pancreas regenerate [34]. We hypothesized that exposing hMSCs, that were in the process of undergoing beta cell differentiation, to this regenerative environment would supply endogenous pancreatic differentiation factors thus completing the differentiation cascade in these transplanted cells. Since mice receiving hMSCs did not achieve normoglycemia as was reported previously in the monocyte derived cells [33], it is possible that the transplantation site may not have been optimal for stem cell differentiation. Transplanting directly into the pancreatic remnant immediately following partial pancreatectomy may have exposed cell grafts to digestive proteins and cytotoxic inflammatory mediators activated by the excision procedure.

These detrimental factors may have superseded the beneficial effects of endogenous betagenic growth factors released by the regenerating pancreas. The kidney capsule has been shown to be an effective transplant site for transplanting islets in the small animal model of diabetes [25], with cells surviving and proliferating within this locale. Although there are not regenerative factors released from the pancreas within the kidney capsule, it would be worthwhile to determine in future work the efficacy of such a transplantation site on hMSC beta cell differentiation.

In summary islet-like clusters, with genetic expression of pancreatic endocrine cell markers, can be derived from human MSCs. Furthermore, following transplantation, hMSCs generate c-peptide which indicates that pancreatic differentiation has been induced. It therefore seems possible that this highly prolific and accessible stem cell source may eventually be used to address the shortage of human islets for widespread islet transplantation making this treatment option a widespread therapy for type 1 diabetes mellitus.

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CHAPTER IV

MOBILIZED PERIPHERAL BLOOD AND UMBILICAL CORD BLOOD ARE POOR SOURCES OF MESENCHYMAL STEM CELLS FOR PANCREATIC BETA CELL DIFFERENTIATION

IV-A) INTRODUCTION

The Edmonton Protocol for islet transplantation [1] provides a new means of treatment for patients with type 1 diabetes. This protocol involves harvesting pancreatic tissue from cadaveric donors and infusing the isolated beta-cell fraction into the portal vein of diabetic patients. This procedure has proved successful with 80% of patients no longer requiring daily insulin injections 1 year post transplantation [1]. Despite the success of this protocol its widespread applicability is limited by the shortage of donor islet tissue and the need for long term immunosuppression in recipients. If islet transplantation is to become a widespread form of therapy for type 1 diabetics, the supply of human organs will be a major limitation, thus alternative tissue sources are being considered. The differentiation of mesenchymal stem cells into insulin-producing beta-cells would alleviate the shortage of human donor tissue.

Mesenchymal stem cells (MSCs) represent < 0.01% of nucleated cells in the bone marrow and provide the stromal support system for hematopoietic stem cells [2]. While these cells represent a minor fraction of the bone marrow, they can be harvested and expanded in culture with high efficiency and have been shown to be able to differentiate into non-hematopoietic cells thus making them potential candidates for differentiation into β-cells. Ianus *et al.* have shown that unfractionated bone marrow, administered intravenously, can migrate to the pancreas and differentiate into insulin-secreting cells [3]. The bone marrow cells composed 1.7-3% of the islet cells and these cells were shown to be glucose responsive with an insulin secretory response similar to control islets. Others have shown that grafts of bone marrow cells which had been transdifferentiated into insulin-producing cells prior to transplantation can reverse diabetes when implanted beneath the kidney capsule of diabetic mice [4]. Further studies have shown that it is the mesenchymal stem cell fraction within the bone marrow that is capable of differentiating into insulin secreting cells [5;6].

If blood sources could yield appreciable numbers of MSCs they could be an accessible, noninvasive source of adult stem cells for islet β-cell differentiation. The question remains whether mobilized adult peripheral blood and umbilical cord blood contain mesenchymal stem cells. Some groups have reported that these sources do contain MSCs [7-9], while others have reported that they do not [10]. Both bone marrow and umbilical cord blood (UCB) have also been shown to contain nestin positive cells [11]. Nestin is considered a neuroepithelial marker and is expressed by some progenitor cells of the pancreas in rat and human pancreatic islets [12]. It is possible that nestin positive cells from these extra-pancreatic sources may be able to differentiate into pancreatic beta cells under the proper conditions. Furthermore, UCB cells have been shown to express markers and transcription factors considered important for beta cell differentiation [13], suggesting that cells from this source may be able to differentiate towards the beta-cell fate.

Traditional approaches toward stem cell differentiation have attempted to direct differentiation by the administration of growth factors. A more direct approach involves transducing cells with genes, such as transcription factors, known to be instrumental during development. Neurogenin 3 (ngn3) is an important transcription factor expressed during beta cell development as islet progenitors mature into endocrine cells of the islet [14]. Ectopic expression of ngn3 in adult pancreatic duct cells has been shown to result in the appearance of endocrine differentiation markers, specifically insulin [15]. Transplantation of stem cells following pancreatic injury, induced by chemicals such as streptozotocin, has also been shown to be an effective strategy for inducing beta cell differentiation [16]. This approach relies upon the regenerative environment within the pancreas, which is generated following injury in the rodent, to provide regenerative growth factors at the dose and time that is required for beta cell regeneration.

The aim of this study was to assess mobilized peripheral blood (mPB) and UCB as potential sources of stem cells for beta cell differentiation. To achieve this aim this study consisted of two purposes; (1) to characterize adherent cells from mPB and UCB for MSC markers to define if these cells express typical cell surface markers known to be expressed on bone marrow derived MSCs and (2) to establish if adherent cells from these sources could be induced to differentiate towards the pancreatic beta cell fate using adenoviral transduction with ngn3.

IV-B) METHODS

Cell Culture

This work was approved by the Health Research Ethics Board at the University of Alberta. Human umbilical cord blood (UCB) and GM-CSF mobilized peripheral blood (mPB) were obtained following informed consent from the Alberta Cord Blood Bank (a gift from Dr. John Akabutu) and the Edmonton Stem Cell Laboratory of Canadian Blood Services, respectively. mPB samples were obtained from the leukopheresed product. Samples were centrifuged and the plasma and buffy coat fractions retained and mixed at a 1:1 ratio with isolation buffer [12.5% Anticoagulant (sodium citrate solution USP, Baxter Corporation, Toronto, ON, Canada) and 5% FBS in PBS]. The mixture of sample and isolation buffer was layered over Ficoll (Ficoll-Paque™ Plus, Amersham Biosciences) and centrifuged. When red blood cells remained within the mononuclear cell fraction, the sample was mixed with red cell lysis buffer (Ammonium Chloride Solution, Stem Cell Technologies, Canada). Following separation, the mononuclear cell fraction was washed with isolation buffer and cultured at a density of 14 x 10^4 cells/cm^2 in tissue culture-treated 150mm plates (mPB) or 12-well plates (UCB) with Mesencult® MSC basal media containing MSC Stimulatory Supplements (Stem Cell Technologies, Canada). Culture dishes were maintained at 37°C (5% CO_2, 95% air) in humidified air with culture media changed every 2 days and removal of nonadherent cells. UCB and mPB samples were cultured in conditions similar to those used for hMSCs [17] to promote growth of this cell fraction. Bone marrow derived MSCs were obtained from

Cambrex Bioscience Walkersville Inc (Walkersville, MD, USA), cultured with Mesencult® growth medium with Stimulatory Supplements (Stem Cell Technologies, Vancouver, BC, Canada), and used as a positive control.

Characterization of Cell Preparations

Adherent cultures (mPB n = 6) were grown for up to 4 weeks. Cell morphology was characterized by phase contrast microscopy using a Zeiss Axiovert 25 inverted fluorescent microscope (Carl Zeiss Canada, ON, Canada) with a Coolsnap camera and IP lab software (Scanalytics inc., Fairfax, USA). Weekly, cells were harvested and characterized by flow cytometry using antibodies (dilution) for CD13 (3/50), CD29 (3/50), CD45 (3/50), Glycophorin A (1/10) (Caltag Laboratories, Burlingame, CA, USA), CD90 (1/50), CD34 (2/5) (BD Biosciences, Mississauga, CA, USA), CD44 (1/10) (Santa Cruz Biotechnology, CA, USA), CD49b (1/10) (Sanquin, Amsterdam, The Netherlands), CD117/c-kit (1/5) and, nestin (1/50) (Chemicon International, Temecula, CA, USA). Cells were fixed with cold 1% formaldehyde (BDH Laboratory Supplies), washed once with PBS and stained with primary antibodies prior to analysis with a FACS Calibur machine (BD Biosciences, Mississauga, Canada) and Cell Quest Pro software and compared to the isotype control. Two samples of mPB were also FACS sorted to negatively select for GlyA$^-$/CD45$^-$ cells using the ELITE sorter (Coulter, Hialeah, FL, USA).

Adenovirus Infection

UCB (n=4) and mPB (n=2) cells were transduced with replication-deficient adenovirus containing cassettes for enhanced green fluorescent protein (eGFP) and murine ngn3 driven by separate CMV promoters (gift from Dr. Harry Heimberg, Free University of Brussels, Belgium) using a modification of a previously described protocol [15]. The eGFP vector provided visual recognition of infected cells and the infection efficiency of experiments was determined by the degree of GFP positive cells in culture following infection. The gene vector for murine ngn3, a transcription factor expressed in the developing beta cell, was administered to cells as a means by which to promote activation of this differentiation pathway in stem cells present within the blood samples. Adherent cells were removed from culture plates using 0.25% trypsin-EDTA and a cell scraper. Cells were washed in HBSS (Hanks Balanced Salt Solution, Sigma), re-suspended in Hams-F10 media and plated in suspension in 5mm non-tissue culture treated plates with 5 mL media. Cultures were infected at a multiplicity of infection (MOI) of 50 with the adeno-ngn3 virus for 4 hours at 37°C, and were subsequently washed two times with media to remove virus particles and re-cultured in Mesencult. Preliminary infection efficiency was judged by visualization of GFP expression on a Zeiss Axiovert 25 inverted fluorescent microscope (Carl Zeiss Canada, ON, Canada) with a Coolsnap camera and IP lab software (Scanalytics inc., Fairfax, USA). Twenty days post-infection mPB cells were removed from culture plates with a cell scraper, centrifuged and sampled for RNA. mPB cells were also infected with adeno-GFP at titrations of MOI 100, 250 and 500 for 24, 48 and 72 hours to determine optimal infection conditions. UCB samples were harvested at days 4, 7 and 12 and mRNA analyzed by RT-PCR.

RT-PCR

RNA samples were pelleted in 2 mL Eppendorf tubes and re-suspended in 1 mL Trizol reagent (Gibco). Samples were frozen at -80 °C prior to RNA extraction using manufacturer's protocol (Gibco). For RT-PCR analysis cDNA was constructed from 1 μg mRNA with 10 units (200 U/μl) Superscript reverse transcriptase in 1X buffer containing 0.1M DTT, 0.5mM dNTPs, and 0.02 μg/μl oligo dT15. For each sample, 1 μl of cDNA was used per 25 μl reaction along with 1X PCR buffer, 2 mM MgCl, 0.2 mM dNTPs, 1 unit taq polymerase (5 U/μl), and 0.5 μM of each primer pair. All chemicals/reagents were from Gibco/Invitrogen. For all PCR reactions, 35 cycles were performed, with 30 second denaturation at 94°C, 30 second annealing at 58°C, and 30 second extension at 72°C. Final polymerization was at 72°C for 10 minutes followed by a 4°C hold. Products were separated on a 2% ethidium bromide stained agarose gel and images captured on Alpha Digidoc software (Perkin-Elmer, Boston, USA). Primer sequences used are detailed elsewhere [18]. Primers were chosen to span intron/extron boundaries to rule out the possibility that amplification products were derived from contaminating genomic DNA. A previously-characterized enriched human islet preparation was used as a positive control for genes examined, negative controls consisted of Sigma water (Sigma, Oakville, ON, Canada) in place of experimental cDNA.

Animals and transplantation experiments

Eight days post-ngn3 infection UCB cells were transplanted to C57BL/6-rag1 knockout mice (n=3) for *in vivo* differentiation experiments. Prior to transplantation animals were injected with 88 mg/kg Streptozotocin to induce pancreatic damage and promote pancreatic regeneration. Mice were anesthetized with halothane and cells were infused i.v. via tail-vein injection. Mice were monitored for blood glucose (OneTouch Ultra™ blood glucose monitors, Lifescan inc., Milpitas, CA) one day post-transplant and weekly thereafter. Forty-one days post transplant mice were sacrificed via cervical dislocation and the following organs were harvested for RT-PCR analysis for the detection of human cells; bone marrow, heart, liver, spleen, brain, kidney, mesenteric lymph nodes, and pancreas. Prior to excision the pancreas was distended with collagenase solution injected via the bile duct. The pancreas was then removed and islets were isolated by collagenase digestion and Ficoll density gradient centrifugation to separate the islet cell fraction from the ductal and exocrine cell fraction [19]. Each cell fraction was analyzed separately via RT-PCR.

IV-C) RESULTS

Morphological and Phenotypical Characteristics of mPB and UCB Cells in Culture

UCB and mPB cells cultured with Mesencult and stimulatory supplements acquired characteristic MSC-like morphological appearance (Figure 4-1) with fibroblastoid-shaped cells that adhered to the culture surface much like MSCs isolated from human bone marrow. With long-term culture (2 weeks for UCB and 4 weeks for mPB) however the adherent cells did not reach confluence as is characteristic for BM-derived MSCs showing that these blood derived cells have poor growth potential (Figure 4-1).

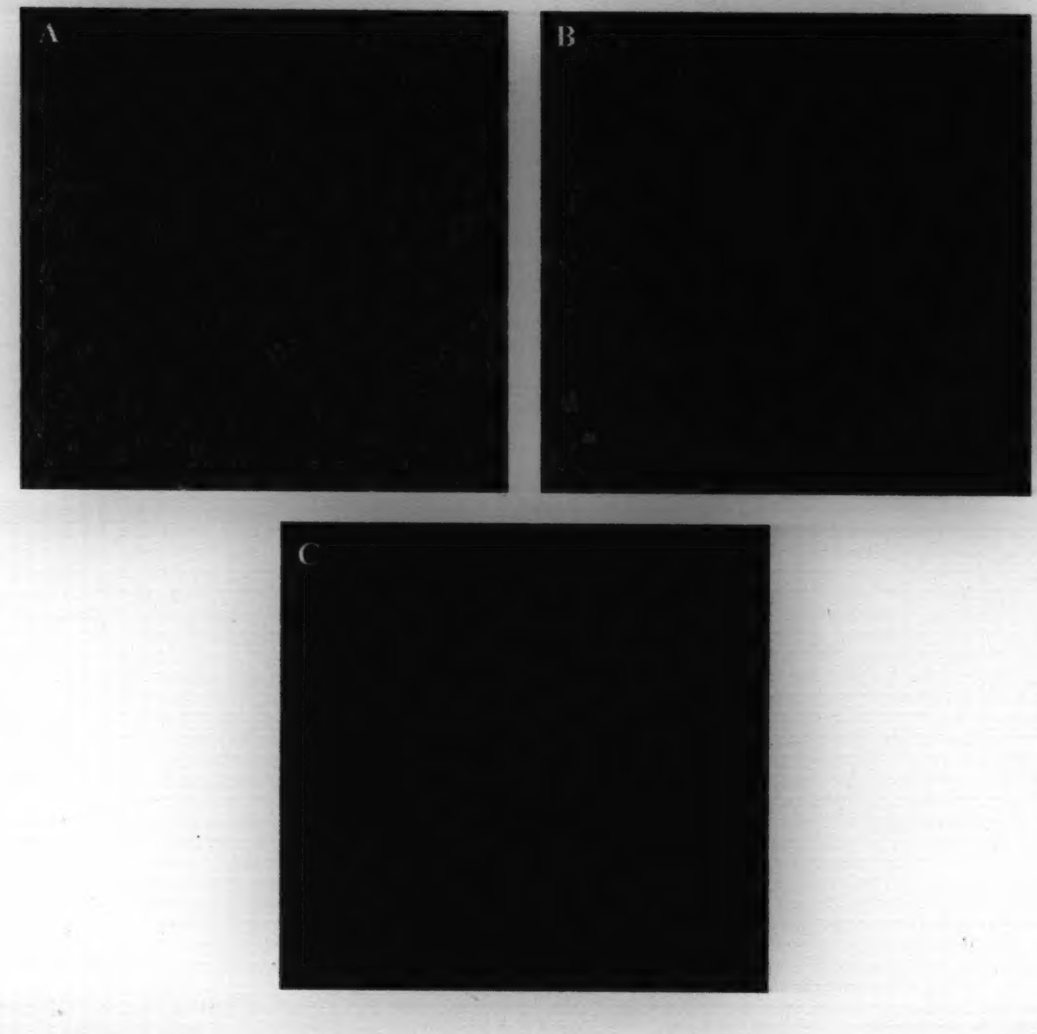

Figure 4-1: Adherent Cell Morphology. (A) Cultured mPB, day 6, (B) Cultured mPB, day 19 (C) Cultured bone marrow derived MSCs, day 5. All culture plates contain elongated spindle shaped cells characteristic of MSCs.

Analyses of mPB samples by flow cytometry revealed that the expression of MSC surface markers CD90, CD44, CD49b and CD29 were lost with time in culture (Table 4-1), indicating that cells with MSC phenotype had poor growth potential and were not sustained in culture. The pan-hematopoietic marker CD45 was maintained and by 3 weeks culture these cells composed 63% of cultured cells indicating that the leukocyte fraction of cells is preferentially maintained with long-term culture. Negative selection of two mPB samples by FACs sorting to select for CD54$^-$/GlyA$^-$, non-hematopoietic cells showed that this double-negative population composed only 1.59% (experiment 1) and 0.06% (experiment 2) of the total cell population. These cells may represent the mesenchymal cell population in mPB that has been described by others [20;21].

Table 4-1. Effect of Longterm Culture on Mobilized Peripheral Blood Cell Composition

	Cellular Proportion (% of Total Cells Analyzed)				
	Weeks in Culture				
Cellular Marker	0	1	2	3	4
CD90	**0.01±0.06 (2)**	**0±0 (4)**	**0 (1)**	**0±0 (4)**	**0±0 (2)**
CD44	**45.67±33.99 (2)**	**7.55±2.32 (6)**	**11.48±6.99 (7)**	**0±0 (5)**	**0±0 (2)**
CD49b	**45.03 (1)**	**11.87±3.28 (6)**	**7.10±3.24 (7)**	**0±0 (2)**	**n/a**
CD13	**15.19 (1)**	**5.32±1.52 (6)**	**1.66±0.68 (7)**	**15.45±15.73 (4)**	**0.50±1.91 (2)**
CD29	**14.16 (1)**	**2.94±1.03 (6)**	**6.20±3.66 (7)**	**21.90±17.73 (4)**	**3.79±5.14 (2)**
CD34	2.25±0.61 (2)	4.63±1.38 (6)	7.59±2.86 (7)	2.95±4.63 (2)	n/a
CD45	95.24±0.79 (2)	85.85±3.41 (6)	62.68±7.94 (7)	62.91±11.55 (5)	12.67±0.41 (2)
CD117(ckit)	18.10±6.43 (2)	20.97±3.05 (6)	5.20±1.74 (7)	1.91±0.96 (5)	0.34±0.32 (2)
Nestin	6.47 (1)	6.14±0.66 (5)	27.55 (1)	4.30 (1)	n/a
GlyA	14.37 (1)	47.72±5.66 (5)	15.70± 5.31 (6)	2.01±1.09 (4)	3.55 (1)

Values are means±SEM (n). Mobilized peripheral blood samples were analyzed with the indicated antibody panel and flow cytometry. Bold lettering indicates cellular markers characteristic of MSCs. n/a: not measured.

Transduction and *in vitro* assessment of UCB and mPB cells

Transduction experiments were performed to determine the differentiation potential of cells from UCB and mPB. UCB samples were infected within the first week of cell culture with adeno-eGFP, ngn3, characterized by fluorescent microscopy and harvested at 4, 7 and 12 days post-infection for RNA. The infection efficiency was ~10-20% of the adherent cells (Figure 4-2), and adherent cells within the cultures that were positive for GFP changed their morphology and developed cellular projections (Figure 4-3). RT-PCR analysis at day 4 post infection showed that cells did not contain mouse ngn3 (the viral vector), nor did they express mRNA characteristic of developing beta-cells; Nkx6.1, Pax6, Pax4, NeuroD or Pdx1. Subsequent samples collected on day 7 and day 12 were analyzed for mouse ngn3 and were negative for this marker.

With mPB the infection efficiency was only ~1% of adherent cells. Adherent cells were cultured for 20 days after which phase contrast microscopy showed that cultures contained adherent cells with spindle-shaped morphology and cellular projections (Figure 4-1 B). Based upon the low infection efficiencies observed at a viral dose of MOI 50 for 4 hours, titration experiments in which mPB was infected at MOIs of 100, 250 and 500 for durations of 24, 48 and 72 hours were performed to determine whether infection efficiencies could be increased with these viral doses. Flow cytometry analysis of these cultures 48 hours post-infection showed that infection efficiency remained at ~1% or less in all titration conditions.

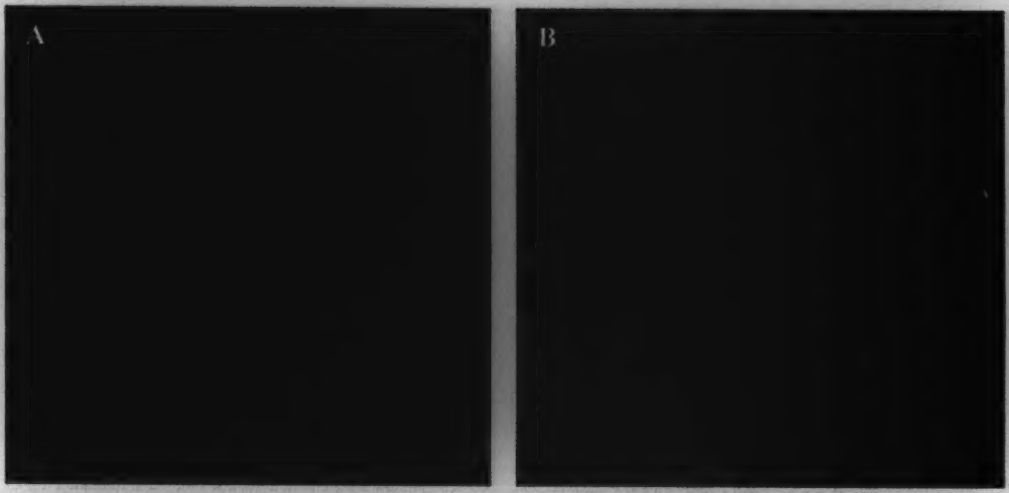

Figure 4-2: Adenoviral Infection of UCB and hMSCs. (A) UCB after ngn3-adenovirus infection at MOI 50. (B) Bone marrow derived MSCs after ngn3-adenoviral infection also at MOI 50, showing high infection efficiency as determined by GFP expression.

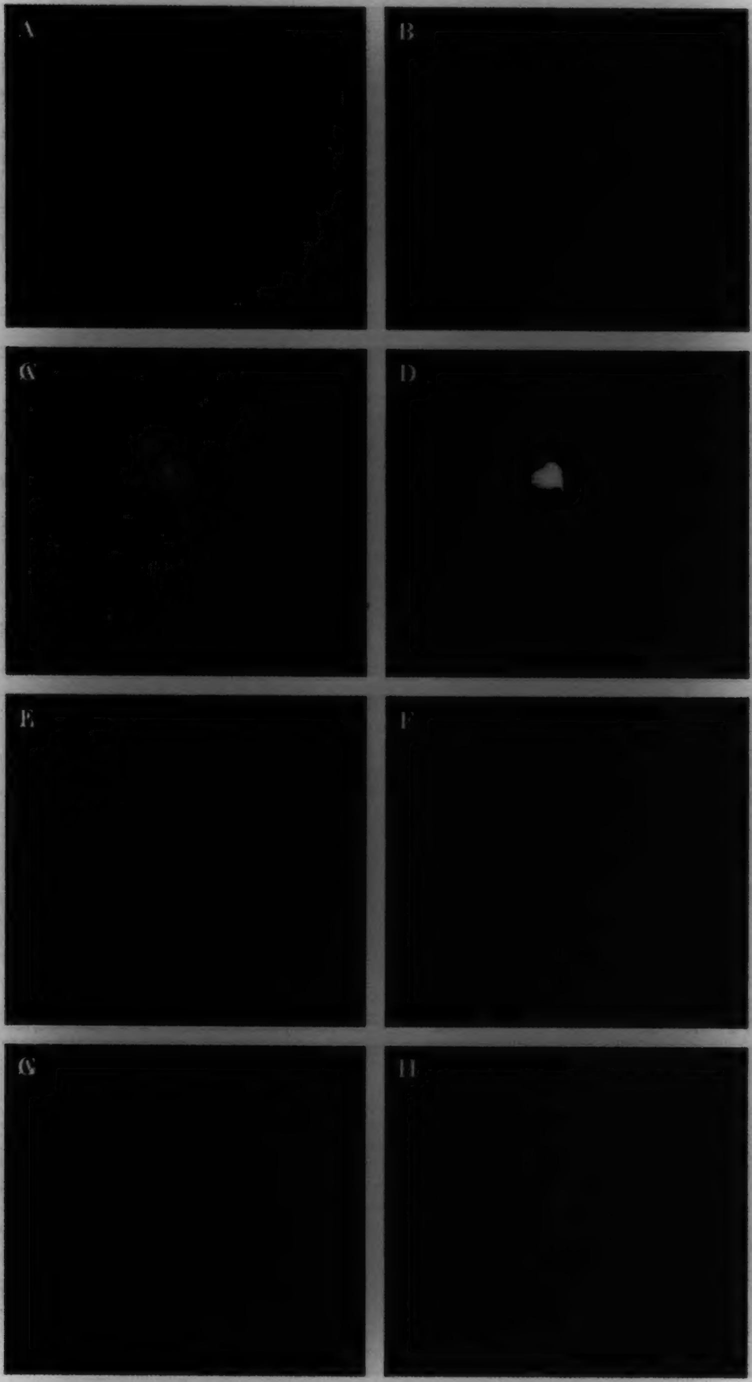

Figure 4-3: Fluorescent microscopy of infected UCB. Representative light (A,C,E,G) and fluorescent (B,D,F,H) micrographs showing infection efficiency and cell morphology at 48 hrs (A-D), 7 days (E,F) and 12 days (G,H) post infection.

In vivo transplantation of UCB cells

In order to assess whether UCB expressing GFP but not the beta-cells markers tested could home to damaged tissue and differentiate into insulin secreting cells, transduced cells were infused intravenously into immune deficient partially diabetic C57BL/6- rag -/- mice. Mean blood glucose at the time of transplant was 12.75 mmol/L and glycemia did not change appreciably over 41 days post transplantation. Despite moderately elevated blood glucose levels, which represent partial damage to the pancreatic beta-cells, mice survived and were healthy during the post transplant period. Mice received 0.7 x 10^6 cells via tail vein injection. Although others have reported infusing up to 200 x 10^6 UCB cell retro-orbitally (RO) [22], we observed that infusing high doses of cells (> 1.0 x 10^6) via tail vein injection resulted in immediate death post-administration, most likely caused by asphyxiation due to cellular infiltration within the lungs. Forty-one days post infusion, multiple organs were harvested and assessed for the presence of human cells via RT-PCR detection of β2 microglobulin. No evidence of mRNA from human UCB was found in any organ assessed in this study. Similar findings were noted by Ende *et al.* in most mice, except for 2/5 mice receiving 200 x 10^6 cells RO [22].

IV-D) DISCUSSION

In this study we present evidence of three main findings. 1. Mobilized peripheral blood and umbilical cord blood contain adherent cells with typical MSC phenotype. 2. These cells did not express classic MSC surface markers in our culture conditions. 3. GFP was expressed in 10-20% of UCB adherent cells, however these transduced cells did not express ngn3 or transcription factors expressed downstream of ngn3. When adherent cells isolated from UCB were injected intravenously into partially diabetic mice we found no evidence of transplanted cells forty-one days post transplant.

In this study we have presented evidence that the adherent cells from human mPB do not express typical cell surface markers characteristic of BM-MSCs. The cultured cells are similar in morphology to MSCs derived from BM however these cells did not expand in culture to reach confluent monolayers in up to 20 days as is characteristic of BM-derived MSCs. Moreover, characterization of the cell surface markers of long term cell cultures showed that surface markers which are characteristic of MSCs decreased with time in culture. There have been reports that MSCs can be detected in peripheral blood of human patients [20;21], however other studies have reported results similar to those presented here [23]. Lazarus *et al.* also attempted to isolate MSCs from the leukapheresis product of mPB by culturing the adherent cell fraction of the samples [23]. Following several weeks in culture they observed few adherent cells and those present did not develop morphological characteristics of MSCs. These findings are similar to what is reported here in that there were few adherent cells, but in this study we observed that the adherent cells displayed morphological characteristics similar to bone marrow MSCs, but did not show the cell surface marker profile of MSCs.

One approach to induce cellular differentiation is to use adenoviral transduction with genes that are expressed during the development of the cell lineage in question. This study attempted to transduce the adherent MSC-like cell fraction of mPB and UBC with genetically-engineered adenovirus containing eGFP and ngn3 with the goal of inducing cell differentiation towards the pancreatic beta-cell fate. Ngn3 is expressed during beta cell development where its expression is limited to the endocrine cell subset within the pancreas. Previous work has shown that adenoviral infection with ngn3 can induce adult human pancreatic duct cells to convert to insulin-expressing cells [15]. At a viral dose of MOI 50, UCB and mPB showed infection efficiencies of less than 10% as measured by GFP expression. Neering *et al.* have reported that infected UCB and mPB at MOIs of 100 and 200 yield infection efficiencies of 25 and 23% respectively [24]. We therefore tested higher viral loads in our samples, but did not observe increases in infection efficiency.

Lu *et al.* have reported gene transfer in umbilical cord derived MSCs using adenoviral vectors [25]. Although the authors report 70% infection efficiency at one week, by 3 weeks the infected cells compose only 0.13% of cell cultures indicating that those cells that were infected with adenovirus died in culture. It is therefore possible that cells within our cultures were infected with the adenovirus but that these cells died prior to our quantification of infection efficiency using GFP expression. In conjunction with the findings of Lu *et al.* it appears that blood-derived stem cells may be sensitive to adenoviral induced cell death and that other viral vectors such as lentivirus may be needed to transduce these cells.

After intravenous injection of adherent UCB cells into partially diabetic mice, no evidence of the transplanted cells was found forty-one days post-transplant in any of the harvested organs, similar to results reported by Ende *et al.* [22]. In that study, type 1 diabetic mice that were transplanted with UCB cells showed delayed onset of autoimmunity, insulitis and improved blood glucose, and human cells could not be detected in the pancreas or other organs, similar to the results reported here.

In summary, this study shows that, although UCB and mPB contain cells with mesenchymal stem cell phenotype, these cells do not express surface markers characteristic of MSCs, highlighting the need for careful characterization of stem cells from different sources prior to the implementation of differentiation strategies. This approach contributes to the knowledge base of stem cell science, as this relatively new area of investigation develops into cell-based therapies.

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CHAPTER V

THE IMPACT OF THE mTOR INHIBITOR SIROLIMUS ON THE PROLIFERATION AND FUNCTION OF PANCREATIC ISLETS AND DUCTAL CELLS

V-A) INTRODUCTION

The clinical success of islet transplantation has provided an effective treatment for type 1 diabetic patients [1;2]. Key elements for this success are the immunosuppressive protocol and transplantation of sufficient islet cell mass. Sirolimus, tacrolimus, and daclizumab are administered to transplant recipients to prevent islet graft rejection [3]. While this cocktail is free of steroidal drugs, which are known to have diabetogenic side effects [2], these agents may still affect the long-term function and survival of human islet grafts.

Several studies have investigated the effects of these immunosuppressive agents on beta cell function in human cell lines [4], rodent beta cells [5] and human beta cells [6-8]. These studies have shown that high doses of tacrolimus and sirolimus can cause significant exocytosis of cellular insulin [4], inhibition of insulin secretion upon stimulation by glucose [5;8] and induction of apoptosis in both beta and alpha cells [6]. A recent clinical update of patients with long-term islet grafts shows that graft function

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diminishes over a 5 year period [1] necessitating exogenous insulin administration for the maintenance of tight glycemic control. Graft function is maintained as demonstrated by persistent c-peptide secretion but the amount of insulin generated by the graft is no longer sufficient to maintain euglycemia in the absence of usually small amounts of supplemental injected insulin. This reduction in insulin production may be the result of beta cell death coupled with impaired beta cell regeneration.

Finegood et al [9] reported that the average lifespan of a beta cell is 1-3 months. Therefore dynamic beta cell turnover occurs within the pancreas to maintain a critical mass of functional beta cells. This also suggests that beta cell turnover occurs within islet grafts to maintain long-term function after transplantation. The source of pancreatic beta cell progenitor cells remains unknown. Using a genetic lineage tracing approach, Dor *et al.* reported that adult pancreatic beta cells are formed by self duplication rather than differentiation from an undifferentiated stem cell [10]. Others have suggested that pancreatic ductal cells can serve as multipotent progenitor cells and that external stimuli can direct the differentiation of these cells to endocrine, acinar, or mature ductal cell phenotypes [11-15]. Other investigators have shown that stem cells, that can differentiate into beta cells may be within the population of bone marrow or spleen cells [16-18].

The working hypothesis of our group is that ductal cells are the beta cell progenitors within the pancreas, contributing via neogenesis to the generation of new beta cells. We have recently shown that islet recipients who receive greater numbers of ductal cells within islet grafts have better graft function at 2 years post transplant as measured by acute insulin response to glucose (AIRg) [19]. Since ductal progenitors within the grafts may contribute to longterm graft function then it is important to determine the effect of immunosuppressive agents on these cells. Indeed, long-term immunosuppression may be reducing graft function either by direct drug effects on the beta cells themselves or also by effects on ductal cell progenitors that are transplanted with the islets (ductal cells form 20-30% of islet grafts) [19].

Sirolimus is a non-specific antiproliferative drug administered to patients receiving islet grafts. Sirolimus inhibits the clonal expansion of activated lymphocytes by interacting with the mammalian Target Of Rapamycin (mTOR), resulting in the inhibition of progression from G1 to the S phase of the cell cycle [20]. Since the proteins bound by sirolimus are not unique to T cells it is possible that sirolimus exerts this antiproliferative effect on other cell types. Studies have shown that sirolimus inhibits proliferation of coronary artery smooth muscles, hepatic stellate cells, small cell lung cancer cells, mouse proximal kidney tubular cells and pancreatic cancer cells [3;20-23]. If sirolimus inhibits the proliferation of pancreatic duct cells or even existing beta cells within the islet grafts this drug may be impeding new beta cell formation and thus limit graft longevity and function.

In the present study we have evaluated the effect of sirolimus on the function and proliferation of human pancreatic islet and ductal cells. We have also tested the effects of sirolimus on neonatal porcine islets (NPI). NPIs constitute a potential source of cells for clinical transplantation because of their inherent ability for proliferation and differentiation [24]. Recently, we have also shown the first evidence that allogeneic NPIs can reverse diabetes in a large animal model [25;26].

V-B) METHODS

Cell Preparation

All experimental protocols used in this study were approved by the Research Ethics Board of the University of Alberta.

Human

Human pancreases were removed from cadaveric donors who had previously given informed consent and processed according to previously described protocols [27]. Briefly, pancreata were stored in chilled University of Wisconsin solution before islet isolation. Islet isolation, gradient purification and tissue collection was performed as previously described for human islets [2;28]. For the *in vitro* insulin secretion experiments fresh (n= 3) or cryopreserved (n= 2) (donor ages 24-64 years) human islets were cultured in non-tissue culture treated plates for 2-3 days with RPMI 1640 medium + ITS/BSA with 10% FCS prior to experimentation.

Following islet purification the ductal/acinar fraction was collected and washed three times with Hanks Balanced Salt Solution (HBSS; Mediatech Sigma-Aldrich, Oakville, Canada) supplemented with 0.5% BSA (fraction V, Sigma). This fraction (n = 4; donor ages 11-67 years) was cultured in suspension for 4-6 days with RPMI 1640 medium (Gibco/Invitrogen, Burlington, Canada) supplemented with 1% insulin-transferrin-selenium (ITS)/0.5% BSA. Media was exchanged at day 1 post culture and every 2 days thereafter. At the end of the culture period these preparations are composed of 68 % CK-19, 16 % amylase, and 1 % beta cells [29].

Pig

The isolation method for NPIs has been described [24]. Briefly, pancreases from Landrace-Yorkshire neonatal pigs (1-3 days old, 1.5-2.0 kg body weight) were removed and digested in 2.5 mg/ml of collagenase (Sigma, Oakville, ON). After filtration (500μm), cells were cultured for 6 days in HAMs F10 medium (Gibco, Burlington, ON) containing 10 mM glucose, 50 μm 3-isobutyl-1-methylxanthine (IBMX; ICN Biomedicals, Montreal, PQ), 0.5% BSA, 2 mM L-glutamine, 10mM nicotinamide (BDH Biochemical, Poole, UK), 100U/ml penicillin, and 100μg/ml of streptomycin. Media was replaced every second day. After this culture period the NPI are routinely composed of 35% endocrine and 57% ductal endocrine precursor (CK-7 positive) cells [24].

Mouse

Islets were isolated from BALB/c mice (purchased from The Jackson Laboratory, Bar Harbor, Maine, USA); pancreases were distended with collagenase solution (1mg/ml), surgically removed and digested. Following dextran gradient centrifugation, islets were picked off gradient interfaces, washed with HBSS and cultured overnight in Hams F10 [30].

Cell Expansion and Characterization

After isolation and 4-6 day culture [29] human ductal cells were cultured at ~ 1.5 x 10^5 cells/well with and without 10 or 20ng/ml sirolimus (Rapamune,Wyeth-Canada Laboratories, Markham, Ontario). NPIs (535 aggregates/well) were cultured as experimental cultures with 20ng/ml of sirolimus added to the base media or as control cultures grown in base media alone. All cells were cultured in 12 well tissue culture treated plates with RPMI 1640 (Gibco) and Hepes buffer, sodium pyruvate, 100U/ml penicillin, 100μg/ml streptomycin, 10% FBS, 2-beta-mercaptoethanol and 20ng/ml EGF and bFGF (Invitrogen) to induce cell proliferation. Cell preparations were assessed for cell number by measuring DNA content at time 0 and day 6 of culture. We have previously shown that human islet cells contain 6.6pg DNA/cell [29] whereas NPI cells have 7.1pg DNA/cell [24]. Cellular insulin content and cellular composition of the NPI cultures were also characterized at these timepoints.

Cell Characterization

To determine cell number, aliquots of the cell suspension were measured in triplicate for total cellular DNA content using a Hoefer DyNa Quant 200 fluorometric assay (Amersham Pharmacia Biotech, San Francisco, CA, USA) as described previously [24]. Insulin content was measured after cellular extraction by RIA (porcine) or by ELISA (human) [24].

The cellular composition of NPI and human ductal cell cultures was determined by immunohistochemistry using the ABC-DAB method. Briefly, cellular aggregates were dissociated into single cell suspensions and adhered to histobond slides (Marienfeld Glassware, Bad Mergentheim, Germany). Slides were fixed in Bouin's fixative for 12 minutes before storage at 4°C in 70 % ethanol. Cells were quenched with a 20% H_2O_2/methanol followed by microwave antigen retrieval for cytokeratins 7 and 19 in sodium citrate. Primary antibody concentrations were as follows: 1/1000 guinea pig anti-porcine insulin (Dako, Glostrup, Denmark), 1/50 mouse anti-human CK19 (Dako), 1/200 mouse anti-human CK7 (Dako). Antibodies to cytokeratins 7 and 19 were used as markers for pancreatic ductal epithelial-type cells as previously described [31]. All biotinylated secondary antibodies were obtained from Vector Laboratories (Burlingame, USA) and used at a concentration of 1/200. Single cell counts were performed on 500 cells per sample and percentages calculated.

Apoptosis in control and sirolimus treated human ductal cells was quantified using TdT-mediated dUTP nick-end labeling (TUNEL) staining. Single cells were adhered to histobond slides and fixed as described above. Cells were stained for CK19 to identify ductal cells using mouse anti-human CK19 (1:25; Dako) and labeled using Cy3 conjugated anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA, USA). Afterward, the apoptotic nuclei were labeled with fluorescein isothiocyanate-dUTP (Roche, Laval, Quebec, Canada) using the TdT enzyme (Deadend Fluorometric TUNEL System; Promega, Madison, WI). The stained slides were analyzed using fluorescent microscopy and cells were counted as described above.

Islet Secretory Activity

Human islets and NPIs were suspension cultured in 79cm^2 dishes with 10ml of RPMI + ITS/BSA, 10% FCS (human islets) or Hams F10 (NPIs), experimental cultures included 20ng/ml of sirolimus, control samples were cultured in base media. All cultures were maintained at 37°C (5% CO_2, 95% air) in humidified air for 6 days, with medium changed every second day. Samples were taken at day 0 and day 6 for analysis of DNA and insulin content, glucose stimulated insulin secretion and immunohistochemical composition.

Human islet and NPI secretory response to glucose were determined using static incubation assay [32]. Islet cell aliquots were washed with Hams F10 media without glucose and incubated for 120 minutes in 1.5ml of Hams media with either 2.8 or 20 mmol/liter glucose. The supernatant was then sampled and assayed for insulin using ELISA (human) or RIA (porcine). The insulin content of the supernatant was expressed as a percentage of the total content (i.e. tissue plus medium). Stimulation indices were calculated by dividing the amount of insulin released at 20 mmol/liter glucose by that released at 2.8mmol/liter glucose [24].

In Vivo Studies

Effect of Sirolimus on Mouse Islet Graft Function

Five-hundred one-day cultured, mouse islets were transplanted under the left kidney capsule of halothane-anesthetized diabetic BALB/c mice (purchased from The Jackson Laboratory, Bar Harbor, Maine, USA). Diabetes (glycemia > 8.4 mmol/l) was induced using 275 mg/kg body weight of Streptozotocin (Sigma) injected intraperitonially (i.p.) two days prior to transplantation. Transplanted mice were randomly separated into two groups; control (untreated) and experimental (sirolimus treated). Sirolimus treatment consisted of daily i.p. injection of 0.2mg/kg sirolimus commencing the day following transplant and continuing for 26 days. Blood glucose levels were monitored weekly between 8:00 and 11:00 am. Forty-eight hours prior to graft removal an oral glucose tolerance test (OGTT) [24] was performed on recipients who had achieved normoglycemia (blood glucose ≤ 8.4mmol/l). After a 6h fast, a bolus of 50% dextrose (3mg/g body weight) was administered intragastrically into nonanesthetized mice. Tail vein blood samples were obtained at 0, 15, 30, 60, 90 and 120 min. Nephrectomy of the graft-bearing kidney was performed and the insulin content of the graft was determined after organ extraction by RIA.

Effect of Sirolimus on Native Pancreatic Islet Function

Naïve BALB/c mice were divided into control (n=11) and experimental (n=11) groups. Experimental animals received daily i.p. injection of sirolimus (0.2mg/kg) for 26 days as detailed above and control animals received daily injections of saline. Blood glucose was monitored weekly and OGTT tests were performed at day 26 to assess pancreatic islet function. Pancreases were removed 48 hours post-OGTT and organs were assayed for insulin content. Alternately, post-OGTT, whole blood was collected from a subset of animals (n=4) into EDTA-treated tubes via cardiac puncture and sirolimus content was measured by RIA.

Statistical analysis

Data are expressed as mean ± SEM of n independent observations. Statistical significance was calculated with a two-tailed unpaired Student's t test or a one way ANOVA in the case of multiple comparisons.

V-C) RESULTS

Sirolimus inhibits proliferation of human pancreatic ductal cells and NPIs. Monolayers of human ductal cells were cultured for 6 days in medium containing no sirolimus (control) and either 10 or 20ng/ml sirolimus. Human ductal cells cultured in control media showed a 1.65 fold expansion (increase in cell number) relative to time 0 while sirolimus inhibited the expansion of human ductal cells and caused a decrease in cell number relative to time 0 (Figure 5-1). With sirolimus treatment, cell-expansion was inhibited relative to control by 53% with 10ng/ml and 50% with 20ng/ml sirolimus ($p<.05$). A dose of 20ng/ml sirolimus was maintained in subsequent experiments, based upon the target trough levels in human islet transplantation (12-15 ng/ml for the first three months and 7-10 ng/ml thereafter) and *in vivo* experimental models [1;2].

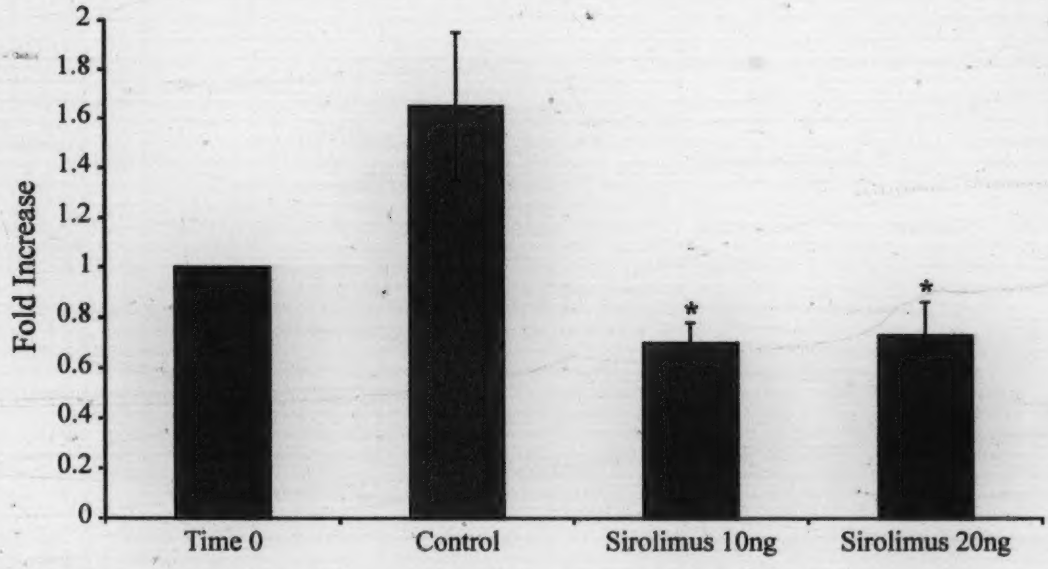

Figure 5-1: The effect of sirolimus on pancreatic ductal cell expansion in vitro. Human ductal cell enriched preparations were cultured in RPMI+ (20ng/mL EGF, FGF and 10% FCS) with and without (control) (10 or 20 ng/mL) sirolimus for 6 days (n=4 in each treatment group). Time 0 represents the initial number of cells in culture while bars for control and sirolimus treated cells represent the change in cell number (fold increase) of cultures relative to the time 0 values. Statistical significance of differences were calculated by one-way ANOVA. *P<0.05 vs. control group.

Decreases in the cell number of sirolimus treated human ductal cells were not due to drug induced cell death as measured by TUNEL staining for apoptosis following 6 days of drug treatment. Control cultures showed that 19.27 ± 3.52 (n=2) percent of CK19 positive ductal cells were undergoing apoptosis, compared with 17.55 ± 7.05 (n=2) percent of CK19 cells in sirolimus-treated cells.

Monolayers of NPIs were cultured on tissue culture treated plates for 6 days in medium containing no sirolimus (control), or 20ng/ml sirolimus. NPIs expanded in number under both control and sirolimus treatment conditions relative to time 0 (Table 5-1). Control treated samples showed a 2.44 ± 0.62 fold increase and sirolimus treated samples showed a 1.77 ± 0.59 fold increase in cell number relative to time 0. Relative to control cultures, sirolimus treatment significantly reduced cell number to 28 % of control ($p<.05$). Examination of cell composition showed that there was differential expansion of cell types; with a decrease in insulin positive cells after 6 day culture and an increase in CK7 positive ductal cells regardless of treatment (Table 5-1).

Table 5-1. Effect of Sirolimus on the Fold Expansion and Cellular Composition of Neonatal Porcine Islets

Condition	Treatment	Percent Positive Cells		Fold Increase
		Insulin Positive Cells	*CK7 Positive Cells*	
Pig	Time 0	14.64±2.12 (5)	29.21±1.93 (5)	1
	Control	7.17±2.07 (5)	47.78±6.80 (5)	2.44±0.62 (9)
	Sirolimus	7.68±2.55 (5)	41.30±14.20 (5)	1.77±0.59 (9)*

Values are means±SEM (n). In each experiment NPIs were cultured with or without (control) 20ng/mL Sirolimus for 6 days and islets were then assessed for cell composition and number as described in Methods. Time 0 represents the initial number of cells in culture. *$p<0.05$ denotes statistical differences relative to control treated samples.

Sirolimus does not alter *in vitro* function of human islets or NPIs. The secretory activity of human islets cultured in the presence or absence of 20ng/ml sirolimus was tested by comparing the percentages of cellular insulin that were released at low glucose (2.8 mmol/l) and high glucose (20 mmol/l). No statistically significant differences were noticed in the amounts of insulin secreted at low and high glucose between treatment groups. The stimulation index, a measure of islet responsiveness to glucose, was not significantly different in time 0, control, or sirolimus treated human samples (Table 5-2). Stimulation indices of control and sirolimus treated cultures were 3.51 ± 1.91 (n=5) and 2.40 ± 0.65 (n=5) respectively, which was slightly (N/S) elevated from time 0 values, 1.42 ± 0.12 (n=4).

The insulin secretory activity of NPIs cultured for 6 days in suspension with medium containing 20ng/ml sirolimus was not different from controls (Table 5-2). Stimulation indices of control and sirolimus treated cultures were 3.01 ± 0.86 and 1.98 ± 0.33 respectively, which was not statistically different from time 0 values of 1.96 ± 0.34.

Table 5-2. Effect of Sirolimus on Glucose Stimulated Insulin Secretion of Human Islets and NPIs

Condition	Treatment	Insulin Secretion (% Content)		
		2.8 mmol/l glucose	20 mmol/l glucose	Stimulation Index
Human Islet	Time 0	7.52±1.82 (4)	10.74±2.91 (4)	1.42±0.12 (4)
	Control	4.96±2.26 (5)	14.71±7.64 (5)	3.51±1.91 (5)
	Sirolimus	5.02±0.99 (5)	13.79±4.76 (5)	2.40±0.65 (5)
Pig	Time 0	2.47±0.52 (4)	4.31±0.14 (4)	1.96±0.34 (4)
	Control	0.46±0.06 (4)	1.35±0.36 (4)	3.01±0.86 (4)
	Sirolimus	0.53±0.05 (4)	1.07±0.24 (4)	1.98±0.33 (4)

Values are means±SEM (n). Stimulation indices were calculated by dividing the amount of insulin released at high glucose (20 mmol/l) by that released at low glucose (2.8 mmol/l).

Sirolimus impairs the function of syngeneic BALB/c mouse islet grafts and innate pancreatic islets. To examine the effect of sirolimus *in vivo*, BALB/c mouse islets were implanted beneath the kidney capsule of diabetic BALB/c mice and sirolimus treatment was administered as described in the methods. Sirolimus had no effect on the daily blood glucose of animals, and animals in both treatment groups were normoglycemic by one week post transplantation (Figure 5-2). OGTT tests demonstrated that mice who received daily injections of sirolimus had elevated blood glycemia at 15, 30 and 60 minutes post bolus administration of dextrose compared to controls. At 60 minutes this trend was statistically significant ($p<0.05$) (Figure 5-2). Insulin content of grafts by organ extraction showed decreased insulin content with sirolimus treatment. However due to large variability these differences were not statistically significant. Control grafts yielded 90.88 ± 47 (n=6) μg insulin/graft whereas sirolimus treated grafts yielded 4.5 ± 2.5 (n=6) μg insulin/graft.

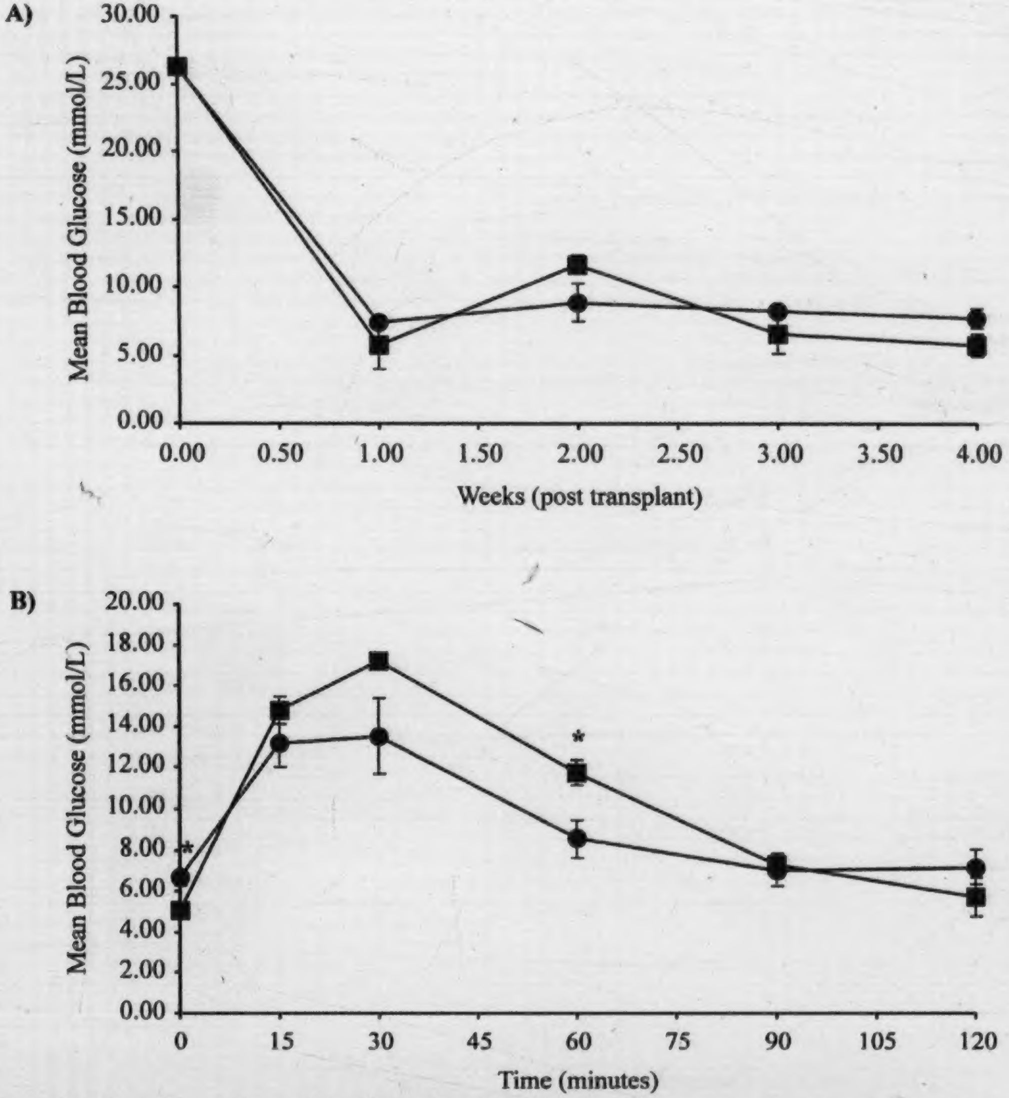

Figure 5-2: a. The effect of sirolimus on daily blood glucose values of syngeneic BALB/c transplants islet grafts. b. Blood glucose values during oral administration of glucose to syngeneically transplanted BALB/c mice, 26 days post transplantation. Comparison of control mice (•; n=3) with those receiving daily injection of sirolimus (■; n=5), i.p. * $P<0.05$ vs. Control.

To examine the effect of sirolimus on pancreatic islet function we injected naïve non-diabetic mice daily with sirolimus (0.2mg/kg) i.p. for 26 days. Sirolimus content of whole blood was 37.03 ± 4.0ng/ml following the injection regime. We found no differences in daily blood glucose levels (Figure 5-3) or pancreatic insulin content between sirolimus treated and control mice. Pancreatic organ extraction for insulin content yielded 27.15 ± 2.9 μg/pancreas in control treated animals and 24.06 ± 1.5 μg/pancreas in sirolimus injected animals. OGTT tests however showed that at 30, 60, 90 and 120 minutes sirolimus treated mice had significantly higher blood glucose than controls at matched timepoints (Figure 5-3). Sirolimus treated mice also showed a tendency towards increased weight gain relative to controls with the difference between start and end weights approaching significance (Control: 0.30 ± 0.3, Sirolimus: 1.49 ± 0.5 p=.078).

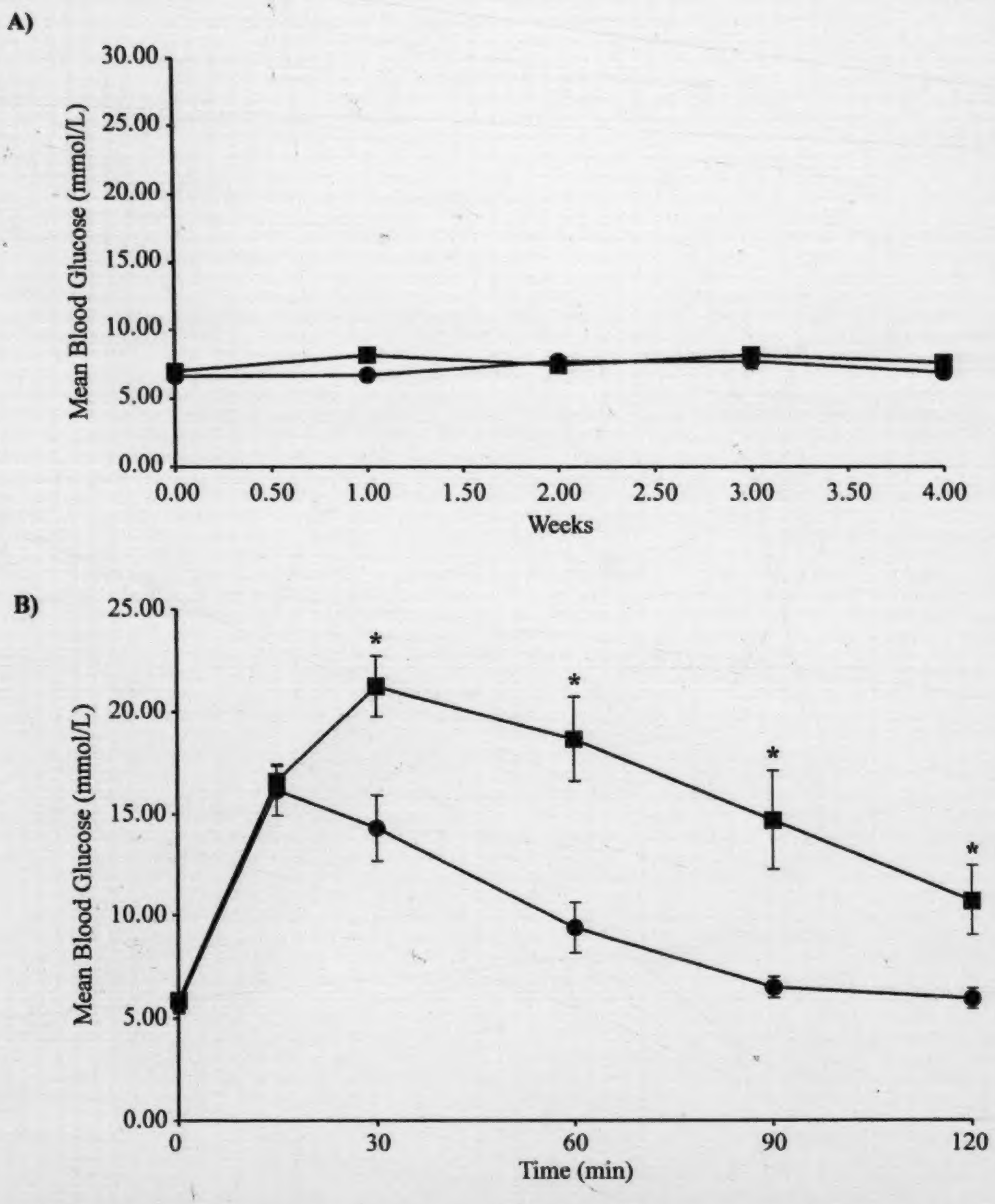

Figure 5-3: a. The effect of sirolimus on daily blood glucose values of naïve BALB/c mice. b. Blood glucose values during oral administration of glucose to naïve BALB/c mice. Comparison of age matched control mice (•; n=11) with those receiving daily injection of sirolimus (■; n=11), i.p. * $P<0.05$ vs. Control.

V-D) DISCUSSION

Prior to this study the effect of Sirolimus on pancreatic ductal cell proliferation was not known. We have evaluated the effect of Sirolimus on ductal cell expansion, islet function and the in vivo function of islet grafts and pancreas. We found that in human ductal cells and NPIs which contain 57% ductal cells, cell expansion was inhibited by 50% and 33% respectively relative to untreated controls. These results are not surprising since Sirolimus interacts with the FKBP intracellular binding proteins. The Sirolimus:FKBP complex binds directly with mTOR and blocks its function. By inhibiting mTOR function, protein translation necessary for cell growth and proliferation is inhibited and sirolimus halts the cell cycle in the G1 phase [33]. We observed that porcine ductal cells are more resistant to Sirolimus than human ductal cells which is of clinical importance for xenotransplantation. If porcine tissue shows enhanced resistance to the negative effects of immunosuppressive drugs such as Sirolimus then the graft longevity of porcine cells may be enhanced compared with human cell equivalents.

We found that Sirolimus treated human ductal cells did not show increased apoptosis relative to controls following six days of drug treatment. Although we did not culture our cells with [^{3}H] thymidine, it has been shown that in human preadipocytes Sirolimus inhibits proliferation as measured by this method [34]. The absence of elevated apoptosis in our study coupled with the absence of proliferation reported by others [34] suggests that the decreased cell number within our Sirolimus treated cultures is likely primarily due to an inhibition of ductal proliferation. When HDCs were cultured with

Sirolimus, cell viability decreased to below that of time 0 (Fig. 5-1), indicating that Sirolimus treatment induced cellular toxicity possibly by an early induction of apoptosis or necrotic cell death prior to day 6, and thus was not detected when we assessed our cultures at day 6. In addition to its anti-proliferative properties, Sirolimus has been shown by others to exhibit cellular toxicity, with a reduction of islet viability as measured by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and Calcein assay occurring at high concentrations (100 ng/ml) [6].

Clinically, patients receiving higher numbers of ductal cells have better long-term graft function despite Sirolimus treatment, indicating that the high number of ductal cells present in islet grafts is sufficient to overcome complete antiproliferative effects of Sirolimus. However sustained treatment with Sirolimus may yield anti-proliferation sufficient to contribute to diminished graft function that is observed with time [1].

To study the systemic effects of Sirolimus, mouse islets were transplanted beneath the kidney capsule of diabetic BALB/c mice. Sirolimus-induced changes were observed in the OGTT profile, with mice showing elevated blood glycemia during the glucose challenge followed by levels returning to normal by the end of the test period.

The observed differences in OGTT profile may be due to poor vasculature caused by inhibition of angiogenesis, altered islet function resulting from ectopic transplantation, inhibition of islet function or reduced ductal proliferation. To determine if differences were due to poor vasculature or ectopic locale we treated naïve mice with daily injections of Sirolimus. These mice had normal daily blood glucose but also showed exacerbated changes in blood glucose regulation upon OGTT testing. The Sirolimus content of whole blood from these mice was 37.03 ± 4.0 ng/ml which is higher than the initial trough target levels of 10-12ng/kg in human islet transplant recipients. We feel however that the high levels of Sirolimus to which the animals are exposed may accurately represent the high peak drug concentrations within the portal circulation, to which human and large animal islet grafts are exposed after being infused into the recipient liver [35]. Similar decreases in insulin response to glucose have been reported in an allograft porcine model of islet transplantation and in the clinical setting of patients who receive islet transplantation [25;36]. In both reports transplant recipients received Sirolimus as part of their immunosuppressive regime and demonstrated elevated glucose following a glucose challenge test.

The sustained hyperglycemia observed during OGTT testing together with increased weight gain in sirolimus treated animals suggests the initiation of insulin resistance in these animals. Sirolimus decreases the phosphorylation of serine 307 on the insulin receptor substrate – 1 protein (IRS-1) in adipocytes thereby inhibiting efficient insulin signaling in human adipocytes and mimicking what is seen with type 2 diabetes [37]. In our work we did not observe any effect on GSIS in human or porcine islets exposed to sirolimus in vitro. Altered OGTT profiles however were observed in vivo when sirolimus was administered to naïve mice and diabetic mice transplanted with syngeneic islet grafts. It is likely that sustained hyperglycemia during OGTT testing resulted from an inhibition of efficient signaling in the adipocyte leading to decreased efficiency of insulin induced glucose sequestration by the adipocyte. It is also known that that sirolimus inhibits insulin induced synthesis of glycogen and protein in muscle [38;39] which may also contribute to the sustained hyperglycemia that we observed in vivo but not in vitro with hyperglycemic challenge.

This study presents convincing evidence that Sirolimus inhibits the proliferation of pancreatic ductal cells and alters GSIS in vivo. Therefore by administering Sirolimus to islet transplant recipients we are likely inhibiting long term graft function by decreasing ductal neogenesis and inducing insulin resistance. Future work in this area will include investigating new immunosuppressive drugs and tolerance induction protocols to replace the use of Sirolimus for effective immunosuppression. Indeed there are many promising new drugs such as sphingosine-1-phosphate receptor agonists or the costimulation inhibitor belatacept which are now undergoing clinical trial that may provide specific T cell inhibition with fewer diabetogenic side effects [40;41].

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CHAPTER VI

VI-A) GENERAL DISCUSSION

The diabetic condition currently affects 194 million people worldwide with this number expected to rise to 333 million by 2025 [1]. Islet transplantation, currently offered only to 'brittle' type 1 diabetic patients, has the potential to be a realistic therapeutic option for all diabetic patients, as all forms of diabetes eventually lead to insulin insufficiency. Problems of islet transplantation including graft failure, immunosuppression, and tissue shortage inhibit widespread distribution of this treatment.

Graft failure and immunosuppression create significant challenges for the long-term success of islet transplantation as demonstrated by the post-transplant follow- up data collected from clinical islet transplantation recipients [2]. Side effects of the immunosuppressive drug regimen such as proteinuria [3] and small bowel ulceration [4] decrease the quality of life for transplant recipients, and the need to resume exogenous insulin in 92.5% of patients [2] also shows that islet grafts are failing with time. With the present day reliance on scarce cadaveric organ donation for islet graft tissue, the number of people who can be treated by this procedure is restricted. Options to remedy this problem include: 1) Ameliorating the yield from the islet isolation procedure [5;6] in order to obtain sufficient islets from one donor pancreas to transplant one diabetic patient – at present two to three donor pancreases are required for one islet transplant recipient [7]. 2) Investigating the therapeutic reality of live donor islet transplantation [8]. 3)

Utilizing xenogeneic sources of islets, such as neonatal porcine islets [9-11]. 4) Inducing the differentiation of insulin secreting beta cells from stem cell sources [12].

The work of this thesis has focused primarily on the issue of stem cell differentiation as a means to remedy the tissue shortages that are limiting widespread islet transplantation. The derivation of new beta cells from adult stem cell sources show promise to address this limitation. Also, should this strategy succeed, the potential for auto-transplantation of differentiated beta cells back into the diabetic patient would be a clinical reality. Such an approach would overcome the need for immunotherapy to circumvent allogeneic graft rejection and could thus expand the procedure to include young diabetic patients who are currently excluded from this therapeutic option due to the potential dangers of lifelong immunosuppression.

A second, separate but related, focus of this work investigated the effects of the immunosuppressive drug Sirolimus on pancreatic islet and ductal cells in human and porcine models. Sirolimus is one constituent of the immunosuppressive drug regimen administered to islet transplant recipients. This drug is an effective immunosuppressive agent as it inhibits the clonal expansion of T cells thereby suppressing the immunogenic response and preserving transplanted islet cell graft [13]. Until now it was not known if Sirolimus exerted an antiproliferative effect on ductal cells within the islet grafts. It was hypothesized, however, that Sirolimus might also inhibit the proliferation of these cells since this drug exerts it effects via ubiquitous cell proteins. Ductal cells are hypothesized to be beta cell progenitors within the pancreas where, following proliferation, they undergo neogenesis to replenish the beta cell population [14;15]. Therefore if Sirolimus inhibits ductal proliferation it is likely that this drug is indirectly contributing to islet graft failure over time.

As a whole, the aim of this work has been to advance the field of islet transplantation by expanding the scientific understanding of the therapeutic potential of adult stem cells for this procedure, and to ascertain the impact that Sirolimus, as part of the islet transplant immunosuppression protocol, might have on adult stem cell function.

With recent reports of the advances in stem cell research for the treatment of disease conditions from Parkinson's [16] to Crohn's [17] disease it seems straight forward in theory to extend these same expectations for the differentiation of adult stem cells into insulin secreting beta cells. In reality however, this aim has been difficult to realize. There have been reports that rodent MSCs can be induced to express beta cell mRNA [18-20] and to secrete insulin in a glucose sensitive way [19]. However these cells still produce less insulin than healthy physiologic pancreatic islet cells and the reproducibility of these protocols has been questioned [21;22]. Work with human MSCs has yielded results which, while being more consistent, have not reported full beta cell differentiation or insulin secretion from the differentiated cells [23;24]. These two published reports working with human MSCs have attempted to induce cellular differentiation by different approaches, and while each show successful induction of mRNA characteristic of beta cells, both works fail to report functionality tests showing that the hMSCs had taken on the beta cell function of glucose stimulated insulin secretion following the differentiation procedures.

The experimental work described in Chapters 2 and 3 of this thesis investigated the potential of multiple differentiation approaches, to induce the differentiation of hMSCs into insulin secreting cells, examining functional changes associated with the differentiation methods as well as morphological transformations and changes in gene and protein expression.

In these chapters evidence is presented of the following main findings. In Chapter 2 evidence was provided showing that: 1. Human bone marrow derived MSCs can be induced to differentiate towards the pancreatic beta cell fate. 2. Adenoviral infection with ngn3 and/or Pdx1 can stimulate MSCs to differentiate towards pancreatic islet cell fate as shown by the expression of mRNA for Pax6, Nkx 6.1, NeuroD, glucagon and insulin. 3. Adenoviral infection with pancreatic transcription factors results in morphological changes within MSC cultures with the formation of Pdx1 positive islet-like clusters. To ascertain the functionality of the differentiated cells we performed *in vitro* glucose challenges and found no detectible insulin secretion. This work is important because it addresses and seeks to define a protocol for successful MSC differentiation into pancreatic beta cells, which if successful will provide a solution for the shortage of beta cells available for widespread islet transplantation for diabetic patients. This study has shown positive results indicating that the conversion of MSC to pancreatic beta cell is possible. Islet-like clusters were formed from the bone marrow stem cells and Pdx1 expression was detected. What is now required are the key elements needed to go from morphological and protein changes to functional changes.

The work presented in Chapter 3 sought to build upon the differentiation approaches of Chapter 2 and demonstrated that: 1. Adenoviral infection of hMSCs with pancreatic genes, timed and sequenced to mimic that which occurs during pancreatic embryogenesis, results in the formation of islet-like cell clusters. 2. hMSCs, differentiated using this protocol, express pancreatic genes both *in vitro* and *in vivo*. 3. Following hMSC transplantation into the regenerating pancreas, human c-peptide was detected in the serum of mice receiving grafts of hMSCs transduced with pancreatic genes. Overall this work showed that emulating embryological events, as a strategy for cellular differentiation, results in enhanced morphological changes and partial functional induction of hMSCs towards the beta cell fate. Together, the work presented in Chapters 2 and 3 of this thesis have advanced the scientific understanding of the impact of multiple differentiation factors on the induction of hMSCs to beta cell differentiation. This work has provided evidence which supports the hypothesis that human bone-marrow derived MSCs may provide an adult stem cell source that may one day be used clinically as a cellular resource for islet transplantation.

If the differentiation of MSCs into clinically suitable beta cells becomes a reality, the derivation of MSCs from the blood instead of the bone marrow would be desirable, due to the ease at which blood samples can be collected relative to bone marrow. There have been reports suggesting that mobilized peripheral blood (mPB) [25;26] and umbilical cord blood (UCB) [27] contain these stem cells. However thorough characterization of the reported 'adherent cells' for characteristic MSC markers has been lacking. This lack of characterization likely contributes to the discrepancies within the literature surrounding whether these blood sources contain MSCs [28-30]. The aim of the fourth chapter of this thesis was therefore to assess mPB and UCB as potential sources of MSCs for beta cell differentiation. In this work we characterized the adherent cell fraction from these cell sources and established if these cells could be induced to differentiate towards the beta cell fate.

The main findings of this chapter were: 1. Mobilized peripheral blood and umbilical cord blood contain adherent cells with typical MSC phenotype. 2. These cells did not express classic MSC surface markers in our culture conditions. It is possible that the mPB in our study did contain MSCs within the fresh samples, but that with long-term culture monocytes/macrophages within the cultures secreted cytotoxic factors that destroyed the MSCs, resulting in a predominantly CD45+ cell population after extended culture which may represent the monocytes/macrophages. In future work, including a negative selection step for the removal of monocytes from the fresh samples may promote MSC growth and yield successful expansion of the MSC fraction from these blood sources. 3. GFP, a marker of adenoviral infection, was expressed in 10-20% of adherent UCB cells. However these transduced cells did not express ngn3 or transcription factors expressed downstream of ngn3, indicating that beta cell differentiation was not induced. When adherent cells, isolated from UCB, were injected intravenously into partially diabetic mice we found no evidence of transplanted cells forty-one days post transplant. Together, the work of Chapter 4 shows that, although UCB and mPB contain cells with mesenchymal stem cell phenotype, these cells do not express surface markers characteristic of MSCs. This evidence highlights the need for careful characterization of stem cells from different sources prior to the implementation of differentiation strategies, and contributes to the knowledge base of stem cell science, as this relatively new area of investigation develops into cell-based therapies.

Finally, in a separate but related study, we investigated the impact of Sirolimus on the proliferation of pancreatic ductal cells and the function of islets. As discussed further in Chapter 5, ductal cells are thought to contain stem cells that are capable of forming new islets – these cells are therefore potentially important for sustained islet graft function. In this work we found that Sirolimus did not alter human or porcine islet function, but did reduce the proliferation of both human and porcine ductal cells. Therefore it is likely that Sirolimus treatment is contributing to the reduced islet graft function that occurs with time [2]. The results of this work provide sound experimental evidence showing the need for improvements to the immunosuppressive regimen administered to graft recipients to ensure sustained islet graft function. Much work is currently being done to examine the clinical reality of strategies such as tolerance induction [31;32]. In addition, there are many new immune cell specific drugs being tested [33;34] which could potentially replace Sirolimus and provide effective immunosuppression while preserving graft function in islet transplant recipients.

VI-B) CONCLUSIONS AND FUTURE DIRECTIONS

This thesis has contributed to the world of scientific knowledge by showing that human MSCs can be induced to differentiate towards the beta cell fate, lending credibility to the hope that these stem cells might one day be used clinically as a cell source for islet transplantation. Furthermore, the work discussed herein has also advanced the knowledge base of stem cell science as a whole by showing that adherent cells from the blood may not be identical to bone marrow derived MSCs and thus these cells might also differ in their capacity for differentiation. Finally, this work provides evidence that the clinical immunosuppressive drug, Sirolimus, inhibits ductal stem cell proliferation. It is also possible that these inhibitory effects of Sirolimus may cause detriment to other stem cells, both transplanted cells, and endogenous stem cells within the body.

Stem cell science as a whole is a relatively new area of work, with many unanswered questions. As stem cell researchers move forward, important consideration for future work include the necessity for defining the cellular characteristics of the stem cells being induced to differentiate. It also appears that the culture conditions of passage number, cell density, culture surface, and growth media have the potential to affect the differentiation fate of stem cells.

I believe that the next chapter of stem cell research will be to fully define the characteristics of these cells and to answer some philosophical questions such as: If in fact adult stem cells exist within the body, what then is preventing these cells from responding and replacing dead or diseased cells? Also, are there physiological inhibitors within the body that are preventing stem-cell induced self healing? It is possible that the most clinically relevant findings resulting from stem cell research will be the identification of endogenous inhibitors of stem cell function, and thus discovering means to promote self healing. Also, for the interdisciplinary field of stem cell research to advance, I believe that there is a crucial need for collaboration and cross-talk between researchers and research disciplines. Cooperation and scientific discussion will likely be the key factors for critical observations and discoveries that will move this new realm of scientific work forward. My hope is that this will be the future of stem cell research, and that by these approaches, significant medical breakthroughs such as the achievement of adult stem cell differentiation into functional beta-cells for the widespread delivery of islet transplantation, will become a reality.

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